Osmolyte protection of *Escherichia coli* and *Salmonella enterica* against inactivation by acetic acid

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Abstract

The combination of acid and osmolytes (salts, sugars) is a common application of 'hurdle technology' used to preserve many foods. Implicit in the hurdle approach is the assumption that as each hurdle is made 'higher', microbiological safety and shelf life will be improved. However, under certain conditions, osmolytes appear to protect *Escherichia coli* and *Salmonella enterica* against inimical acetic acid challenge. This thesis: 1) describes the apparent protection of *E. coli* and *S. enterica* by moderate levels of NaCl and sucrose against inactivation by acetic acid under non-growing conditions; 2) places those observations of protection in context with the published literature, international industry guidelines and Australian contemporary manufacturing processes for cold-filled sauces, dressings and mayonnaises; and 3) investigates possible mechanisms of this protection involving changes in the cell envelope.

The time to a 3-$\log_{10}$ reduction of *E. coli* O157 strain SERL 2, in the presence of acetic acid, was observed to be non-monotonic, i.e. initially decreasing, but then increasing, in response to increasing NaCl concentrations but also to be dependent on pH / acid concentration. Further study demonstrated osmolyte protection against acetic acid inactivation to be conserved among different strains of acid resistant *E. coli* and *S. enterica*, and to be largely independent of osmolyte type (NaCl, sucrose). In addition, simple descriptive models were developed from data available in the published literature. From those models, an increase in time-to-3-$\log_{10}$ reduction with increasing sucrose concentration was predicted for *E. coli*. The models also predicted an increase in the time-to-3-$\log_{10}$ reduction with increasing NaCl for *S. enterica*. From the published data the concentration of NaCl accounted for little of the variability. pH and 1/ absolute temperature were the most important predictor variables, collectively accounting for at least 50% of the variability in the models. Conditions under which osmolyte protection of *E. coli* and *S. enterica* were observed fall within the recommendations of international industry guidelines (the "CIMSCEE Code") for the manufacture of microbiologically-safe cold-filled acid sauces, dressing and mayonnaises. A survey of Australian manufacturers of cold-filled acid products confirmed that contemporary formulation practices included conditions for which osmolyte protection against acetic acid could be expected.

The antibacterial activity of acetic acid is usually explained by weak acid theory, involving diffusion of the undissociated moiety followed by dissociation and cytoplasm acidification, and acetate anion accumulation and toxicity. These proposed mechanisms are ultimately mediated at the level of the cell envelope and, in particular, the cytoplasmic membrane. The most obvious changes due to osmotic stress affect the structure and composition of the cell envelope, suggesting a possible mechanism of protection against acetic acid inactivation, i.e., that non-monotonic inactivation in response to increasing osmolarity in the presence of acetic acid arises from damage to, or changes in, the cell envelope. Improved survival at 'intermediate' (hypertonic) osmolarities could arise from: 1) one type of damage increasing with osmolarity above or below the optima, or 2) one type of damage increasing with increasing osmolarity and a second type of damage increasing with decreasing (hypotonic) osmolarity.

Studies of *E. coli* and *S. enterica* recovery in the presence of bile salts and crystal violet suggested that damage to the outer membrane in the presence of acetic, but not hydrochloric, acid is non-monotonic with increasing osmolarity. Flow cytometry and Three Dimensional Structured Illumination Microscopy (3D-SIM) were used to assess membrane changes in the total population of *E. coli* and *S. enterica.* Substantial outer membrane damage by acetic acid was confirmed in this manner using the fluorescent dyes, hexidium iodide and SYTO® 9, with the latter changing non-monotonically with increasing osmolarity. Outer membrane damage to *E. coli* and *S. enterica* by acetic acid has not previously been reported. Outer membrane damage to *E. coli* was also observed to be substantially slowed by storage at 5°C, correlating with improved survival.

Substantial loss of cytoplasmic membrane integrity, as assessed by flow cytometry with propidium iodide, was not observed. However 3D-SIM showed the development of distinct, brightly SYTO® 9- stained membrane domains in response to increasing exposure time, osmolarity and acidity, suggesting that changes in cytoplasmic membrane structure, if not integrity, did occur. Using 3D-SIM and staining with nonyl acridine orange (NAO), it was shown that the membrane domains were enriched with cardiolipin. Cardiolipin has previously been shown to be involved in the response of *E. coli* to osmotic stress, but production in response to acid, and acetic acid, stress

has not previously been reported for *E. coli* or *S. enterica*. The proportion of cells exhibiting cardiolipin-enrichment increased with increasing exposure time, and responded non-monotonically to increasing osmolarity. The point of minimum cardiolipin-enrichment typically occurred at a lower osmolarity than that for cell inactivation and outer membrane damage, thus correlating minimum inactivation in the presence of acetic acid in response to osmolarity with *some* cardiolipin enrichment.

Cardiolipin enrichment has been previously shown to alter membrane permeability, membrane potential (Δψ) and aggregation of membrane proteins. In this study membrane fluidity, assessed using fluorescence polarisation of the probes DPH and TMA-DPH revealed a complex, dynamic, bimodal response to increasing osmolarity in the presence of acetic acid for both *E. coli* and *S. enterica*. Similarly, assessment of Δψ changes suggested that any advantage of cardiolipin production to cell survival in the presence of acetic acid is complex. Among *E. coli* and *S. enterica* populations exposed to inimical acid conditions for 72h, fewer of the cells enriched in cardiolipin were depolarised. However, at the shortest exposure time of 6h more cardiolipin-enriched than non-cardiolipin-enriched cells were depolarised at higher osmolarities, while more non-cardiolipin-enriched than cardiolipin-enriched cells were depolarised at lower osmolarities. Therefore, determination of the significance of cardiolipin enrichment in the presence of acetic acid on membrane function remains unclear at this point, and requires further investigation.

In conclusion, this study generated novel insights regarding cell envelope changes that accompany exposure to combined acetic acid and osmotic stress, and how these responses are modified by pH and temperature. *E. coli* and *S. enterica* are protected against inactivation by acetic acid by osmolytes, and this protection appears coincident with maintenance of outer membrane integrity and with some increase in the cardiolipin content of the cytoplasmic membrane. It remains a compelling hypothesis that such changes in the cell envelope could contribute to osmolyte protection of *E. coli* and *S. enterica* against acetic acid inactivation.

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1 Introduction and objectives

Most processed foods utilise more than one preservative factor to achieve microbiological stability and safety (Leistner and Gould, 2002). These preservative factors have been termed ‘hurdles’ (Leistner and Rödel, 1976). Their combined influence on microorganisms is termed the ‘hurdle effect’ or ‘hurdle concept’ (Leistner, 1978), and its application in food processing, as ‘hurdle technology’ (Leistner and Gould, 2002). The simultaneous application of acid and osmolytes (salts, sugars) is an example of hurdle technology used in the preservation of a large range of foods, including fermented meats, preserved vegetables, cheeses and cold-filled acid sauces, dressings and mayonnaises (Booth and Kroll, 1989).

Implicit in hurdle technology is the assumption that as each hurdle is made ‘higher’ microbiological safety and shelf life will be improved. However the potential for relatively low concentrations of some food preservatives to act as stimulants to microbial growth has long been noted: Ingram and Kitchell (1967) stated, “as a rule, relatively low concentrations of salt stimulate micro-organisms while higher concentrations inhibit them”. Radford and Board (1995) postulated that one of the possible outcomes of the surge in application of hurdle technology in response to consumer demands, is the formulation of lower salt foods in which NaCl has an inadvertent stimulatory or protective effect on microorganisms in the presence of other preservative agents.

Since its publication in 1992, the commercial production of microbiologically safe and stable emulsified and non-emulsified sauces and dressings containing acetic acid has been guided by the Comité des Industries des Mayonnaises et Sauces Condimentaires de la Communauté Économique Européenne’s Code (the CIMSCEE Code; CIMSCEE, 1992), based on the earlier work of Tuynenberg Muys (1971). The CIMSCEE Code promotes a hurdle technology approach to preservation, using a combination of acidity, acetic acid, sodium chloride, disaccharide (e.g. sucrose) and hexose (e.g. glucose) concentrations to achieve microbiological stability and safety.

Consumer demands for “healthier” products (i.e. less processed and with fewer additives) with improved taste have resulted in a plethora of “gourmet”, “lite”, “calorie-reduced” and “salt-reduced” cold-filled acetic acid-containing products (Sloan, 2004; Pszczola, 2007; Pszczola, 2008). Increasing demand from the food service sector for convenient, pre-prepared ready-to-eat meals and meal components is also changing post-manufacture handling and distribution practices for cold-filled acid products (Sloan, 2004). As formulations and distribution practices move further away from those traditionally used for cold-filled acid products, it cannot be assumed that current manufacturing guidelines remain adequate to address microbiological stability and safety.

Recent studies demonstrate the complexity of antimicrobial interactions between ingredients used in cold-filled acid sauces, dressings and mayonnaises, with observation of effects on spoilage microorganisms that are apparently inconsistent with the hurdle concept. For example, in formulations in which lactic acid was used to replace partially acetic acid, low levels of lactic acid were found to stimulate the growth of the key spoilage yeast *Zygosaccharomyces bailii* (Vermeulen *et al.,* 2008). Further, Dang *et al.* (2009) found that low levels of acetic acid could also be stimulatory to the growth of *Z. bailii*, but only at low temperature.

It has been observed that osmolytes may protect *Escherichia coli* and *Salmonella enterica* against inimical acidity in cold-filled sauces, dressings and mayonnaises. Incorrect predictions of *E. coli* inactivation (i.e. predicting death when survival occurred) from the CIMCEE Code have been noted to be correlated with the concentration of NaCl and sucrose in models of mayonnaise (McKellar *et al.,* 2002). Larson *et al*. (1993) showed that the rate of inactivation of *Salmonella* Heidelberg increased in unsalted rennet whey at pH 4.8 or 5.6, compared with rennet whey salted at 2.4% NaCl. Radford and Board (1995) demonstrated that *Salmonella* Enteritidis was stimulated by ~ 2% (w/v) NaCl in the presence of acetic acid, and Jordan and Davies (2001) showed that the addition of 4 to 6% (w/w) NaCl stimulated growth of *E. coli* in tryptone phosphate broth. Casey and Condon (2002) showed that the addition of 4% NaCl, or fructose at an equivalent osmolarity, improved survival of an enteropathogenic *E. coli* (EPEC) strain O157:H45 (as a model for enterohaemorrhagic *E. coli* (EHEC)) in the presence of lactic and acetic acids. McKellar *et al.*

(2002) developed a probability model to describe the interface between survival and death of *E. coli* O157:H7 in a mayonnaise model system that predicted the survival / death pH interface at 3% acetic acid and 30°C to be reduced from pH 4.5 – 5.0 at 0.5% NaCl to pH 4.0 – 4.5 at ≥1.0% NaCl. Simulations with 2 - 4% acetic acid and increasing levels of NaCl or sucrose showed a generally increasing probability of survival with up to ~ 3.5 - 4.5% NaCl and 4 - 8% sucrose, especially at higher temperatures and lower acetic acid concentrations (McKellar *et al.,* 2002). Lee and Kang (2009) and Lee *et al.* (2010) showed that the addition of 3% NaCl significantly increased the resistance of *E. coli* O157:H7 to acetic acid inactivation in laboratory broths, and pickled cucumber products. In minimal medium the growth rate of *E. coli* has been found to be maximal at ~0.3 OsM (~1% NaCl) and this osmolarity is therefore assumed to represent isotonicity (Record *et al.*, 1998). Thus in the majority of cases reported, the protective osmolyte concentration would initially present a mild to moderate hypertonic environment.

With the increasing use of pasteurised egg products since the 1970s, concerns about the importance of *Salmonella* in cold-fill products declined (Glass and Doyle, 1991). Concerns about EHECs or Shiga-toxin-producing *E. coli* (STEC) rose, however, particularly in light of their acetic acid resistance (Michels and Koning, 2000). These new concerns peaked with an outbreak of *E. coli* O157:H7, involving an estimated 300 people, that was linked to a commercial mayonnaise (Weagent *et al.*, 1994). This outbreak however was later determined to be due to cross-contamination from uncooked meat (Michels and Koning, 2000). Epidemiological evidence and likelihood-of-contamination assessments have since concluded that commercially produced acidic dressings and sauces are not high-risk vehicles for STEC (Erickson *et al.*, 1995). Nonetheless, concerns about contamination of such products with *E. coli* and *Salmonella* remain, and particularly with regards to a number of contemporary manufacturing, consumer and food service practices, including reductions in acetic acid, NaCl and sugar concentrations, the inclusion of fresh ingredients such as herbs, re-use during open shelf-life, and use in the preparation of mayonnaise-based salads (and especially those containing meat). Accordingly, understanding the response of acid-resistant pathogenic *E. coli* and *Salmonella* to various hurdle combinations is important, given their importance to the broader food industry. Indeed, observations that apparently invalidate the hurdle concept deserve further investigation. To understand the potential

of one hurdle to protect against another, it is important first to understand the mechanism by which each hurdle damages the cell.

The antibacterial activity of acetic acid is traditionally explained by weak acid theory, which proposes that acetic acid in its undissociated, lipophilic, form will cross the cytoplasmic membrane and then dissociate within the more neutral cytoplasm, resulting in its acidification and destabilisation of macromolecules (Eklund, 1989). Axe and Bailey (1995) have suggested that, given its lipophilicity, acetic acid may act as an uncoupler, crossing and recrossing the cytoplasmic membrane and thereby dissipating the proton motive force (ΔP). However an alternative and somewhat contradictory hypothesis to explain microbial inactivation by acetic acid is that of acetate anion accumulation and toxicity (Russell, 1992; Diez-Gonzalez and Russell, 1997). In this case, it is assumed that rapid anion efflux is precluded by the barrier action of the lipid tail region of the cytoplasmic membrane.

Casey and Condon (2002) suggested the protective effect of NaCl on inactivation of *E. coli* O157:H45 by lactic acid was principally due to the increase in cytoplasmic pH observed in the presence of NaCl, but also speculated on the possible existence of a sodium-proton antiporter. Kitko *et al.* (2010) proposed that protection by osmolytes against HCl may involve cell volume maintenance, thus avoiding concentration of acid species and a decrease in pH. However Casey and Condon (2002) demonstrated a decrease in cell volume to be correlated with pH maintenance. In another contrast, Hosein *et al.* (2011) have suggested that the lower intracellular pH of *E. coli* in the presence of NaCl and acetic acid may reduce accumulation of acetate anions. Finally, a possible role for Na^+ in acid resistance has been linked to induction of the Gad regulon, which regulates glutamate-dependent acid resistance (Richard and Foster, 2007).

Alone, the most notable effect of non-permeant osmolytes such as NaCl and sucrose is that of plasmolysis, which occurs under hypertonic conditions (Scheie, 1969). In Gram-negative bacteria, the separation of the cytoplasmic membrane from the peptidoglycan (murein) wall and the outer membrane results in the development of plasmolysis spaces, which has been proposed in turn to indicate a decrease in the surface area of the cytoplasmic membrane (Koch and Woeste, 1992). It

has been proposed that this decrease in surface area can arise via a number of different mechanisms, including wrinkling or invagination of the cytoplasmic membrane, and pinching off of regions of the cytoplasmic membrane to form endocytotic vesicles (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998). The presence of osmolytes has also been demonstrated to alter the composition of membranes (McGarrity and Armstrong, 1975; Russell and Kogut, 1985; Romanstov *et al.*, 2008; Romanstov *et al.*, 2009).

It is apparent that all proposed mechanisms of acetic acid inactivation are mediated at the level of the cell envelope, and in particular the cytoplasmic membrane. The most obvious changes brought about during osmotic stress also relate to changes in the structure and composition of the cell envelope. Therefore, a possible and hereto largely unexplored mechanism of protection against acetic acid inactivation is suggested. It is hypothesised that the ability of (initially) hypertonic concentrations of osmolytes to protect against inactivation by acetic acid may arise, at least in part, as a result of a non-monotonic pattern of damage to, or changes in the cell envelope. Improved survival at “intermediate” (hypertonic) osmolarities could arise as a result of 1) one type of damage increasing both with decreasing and increasing osmolarity, or 2) one type of damage increasing with increasing osmolarity and a second type of damage increasing with decreasing (hypotonic) osmolarity

Thus, the aims of this thesis are; 1) to expand understanding of the protective effects of osmolytes on the inactivation of *E. coli* and *Salmonella enterica* by acetic acid under non-growth permissive conditions; 2) to review the relevance of observations of protection (this thesis, and within the published literature) to contemporary manufacture of acetic acid containing cold-filled sauces, dressings, and mayonnaises; and 3) to investigate a possible mechanism of protection of osmolytes against inactivation by acetic acid. Specifically these aims are addressed within the structure of this thesis by:

1. reviewing the literature concerned with the safety of acetic acid containing foods with respect to *E. coli* and *Salmonella*, and the mechanisms by which acetic acid interacts with and inactivates cells, with a particular focus on cell membranes, the potential effects of

osmolytes on acetic acid interactions with membranes, and the survival of *E. coli* and *Salmonella* in the presence of acetic acid under various osmotic and other conditions (**Literature review**);

2. establishing the effect of NaCl concentrations, with and without sucrose, on the inactivation kinetics of an acid tolerant enterohaemorrhagic strain of *E. coli* O157, SERL 2, in the presence of acetic and hydrochloric acids, at different pH levels (**Manuscript 1: *E. coli* survival in acid, salt, and sucrose**);

3. investigation of the effect of sucrose on inactivation of enteric bacteria by acetic acid, in order to determine whether osmolarity is a more fundamental predictor variable than NaCl or sucrose concentration (**Manuscript 2: Protection against acetic acid by hypertonicity**);

4. extending knowledge of the effect of osmolarity on inactivation by acetic acid to other *E. coli* strains, and to acid tolerant strains of *S. enterica* (**Manuscript 2: Protection against acetic acid by hypertonicity**);

5. surveying Australian manufacturers of acid sauces, dressings and mayonnaises to determine contemporary formulation and storage variables and their ranges, especially acid concentration, osmolytes, pH and storage temperature, that might affect the safety of these products, (**Manuscript 3: Cold-filled acid products: survey and pathogen modelling**);

6. compiling published kinetic inactivation data for *E. coli* and *S. enterica* in acid foods, determining the feasibility of modelling the inactivation of these pathogens to meet the needs of contemporary manufacture, and determining the relative importance of predictor variables of relevance to industry, especially acid concentration, osmolytes, pH and storage temperature (**Manuscript 3: Cold-filled acid products: survey and pathogen modelling**);

7. determining the effect of osmolarity, pH and temperature on outer membrane permeability of *E. coli* and *S. enterica* in the presence of acetic acid (**Manuscript 4: Membrane changes with acetic, osmolytes, cold and pH** (including supplementary material))

8. determining the effect of osmolarity, pH and temperature on cytoplasmic membrane integrity of *E. coli* and *S. enterica* in the presence of acetic acid (**Manuscript 4: Membrane changes with acetic, osmolytes, cold and pH** (including supplementary material))

9. determining the effect of osmolarity, pH and temperature on kinetics of cytoplasmic membrane composition changes of *E. coli* and *S. enterica* in the presence of acetic acid (**Manuscript 5: Cardiolipin in *E. coli* and *S. enterica* with acetic**);

10. determining the effect of osmolarity on *E. coli* and *S. enterica* cytoplasmic membrane potential and fluidity in the presence of acetic acid (**Manuscript 5: Cardiolipin in *E. coli* and *S. enterica* with acetic**).

2 Literature review

2.1 Abstract

Literature discussing hurdle technology for the production of cold-filled sauces, dressings and mayonnaises that are considered microbiologically safe with respect to pathogenic *Escherichia coli* and *Salmonella enterica* was reviewed. While published challenge studies generally demonstrate the effectiveness of acetic acid for the inactivation of *E. coli* and *S. enterica*, a number of studies clearly suggest that increasing the concentration of NaCl or sugar in the presence of acetic acid (especially particularly when levels are “low”) improves cell survival. The key mechanisms of acetic acid inactivation of bacteria are generally considered to be intracellular acidification following passive diffusion of the undissociated molecule and dissociation in the cytoplasm, uncoupling, and / or anion accumulation. All of these mechanisms are ultimately related to the permeability of the cell envelope, and especially the permeability of the cytoplasmic membrane to the various acetic acid species. However, very little work has been published on changes in the cell envelope (i.e. outer membrane and cytoplasmic membrane) of *E. coli* or *S. enterica* in response to acetic acid stress under non-growth conditions. With increasing osmolyte concentrations, the most obvious effects are manifest at the cytoplasmic membrane. The potential for acetic acid and osmolyte effects to interact, including antagonistically, at the level of the cell envelope are thereby highlighted.

2.2 Hurdle technology for cold-filled acid sauces

2.2.1 Introduction

Most processed foods rely on more than one preservative factor to achieve microbiological stability and safety (Leistner and Gould, 2002). These preservative factors have been termed ‘hurdles’ (Leistner and Rödel, 1976), the effect of their combination on microorganisms is termed the ‘hurdle effect’ (Leistner, 1978), and the application of the ‘hurdle concept’ to food technology

is termed 'hurdle technology' (Leistner and Gould, 2002). Effective application of hurdle technology requires that microorganisms that are present in a food are unable to overcome the hurdles present, which may operate sequentially (e.g. cooking, followed by refrigeration) or, more often, in unison as part of the food formulation (Leistner and Gould, 2002). The preservative combination of low pH (acid) and low a_w (e.g. NaCl and sucrose) is a classic example of hurdle technology, and is used in the formulation of a large and diverse range of foods, including acidic condiments and dressings, fermented meats, cheeses and preserved vegetables (Booth and Kroll, 1989).

Inherent within the hurdle approach is the assumption that as more hurdles are added (e.g. acid and salt), or as each hurdle is made 'higher' (e.g. increasing concentrations of salt), there will be a greater microbial inactivation (or inhibition) (Lee and Kang, 2009). In some cases, the overall inactivation level may represent only the additive effects of the individual hurdles, but in other cases the overall 'kill' may be synergistic (Leistner and Gould, 2002). Synergistic inactivation can occur when preservative factors have different targets within the microbial cell, thus disturbing homeostasis in several cellular systems simultaneously; for example cell membranes, DNA replication, and various enzyme systems (Leistner, 1994). Leistner and Gould (2002) proposed that a better understanding of the mechanisms of action and interaction of preservative hurdles can form a powerful, logical basis for improvements in food preservation technology, encompassing not only the microbiological safety and stability of foods, but also their sensory and nutritional quality (Leistner, 1994). The primary goal of hurdle technology is to reduce the amounts of individual preservatives (e.g. acid) and the severity of individual processes (e.g. heating), even though the number of preservatives and processes may increase to enable multi-target preservation (Leistner and Gould, 2002).

2.2.2 Microbiology of acid sauces, dressings and mayonnaises

The microbiological stability and safety of sauces, dressings and mayonnaises is well-understood, and is typically described with respect to the severity of two key hurdles: acidity (pH), usually predominantly conferred by the addition of acetic acid, and thermal processing (Michels and

Koning, 2000). As pH decreases, microbiological safety and stability can be achieved without thermal processing; conversely, as thermal processing increases, microbiological safety and stability can be achieved at higher pH.

For cold-fill products the key target spoilage microorganisms are yeasts and lactic acid bacteria and, although generally of a lesser concern, moulds (Michels and Koning, 2000; ICMSF, 2005). For warm-fill products, lactic acid bacteria are the key target spoilage microorganisms (Smittle and Flowers, 1982; Michels and Koning, 2000). For hot-fill and acid pasteurised sauces, if pH is increased while the concentration of acetic and other acids is decreased, the more heat-resistant spore-former *Bacillus coagulans* becomes more important that the less heat resistant *Clostridium pasteurianum* (Michels and Koning, 2000). At pH >4.6, the main microorganism of concern for low acid sauces is the neurotoxigenic spore-former *Clostridium botulinum* (Michels and Koning, 2000).

Among the spoilage yeasts, a number of genera and species have been implicated in the spoilage of cold-fill products, including *Pichia membranaefaciens*, *Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae*, *Candida magnolia*, *Zygosaccharomyces bisporus*, *Rhodotorula mucilaginosa* and *Debaromyces hansenii* (Michels and Koning, 2000). However, *Zygosaccharomyces bailli* is generally considered the main spoilage microorganism of acidified food and beverages (Erickson and McKenna, 1999), due to its exceptional resistance to low a_w, low pH, organic acids (including acetic acid), and the weak acid preservatives sorbic and benzoic acid (Praphailong and Fleet, 1997). Among the lactic acid bacteria, *Lactobacillus fructivorans*, *Lactobacillus plantarum* and *Lactobacillus buchneri* are considered the principal spoilage species (Smittle and Flowers, 1982; Michels and Koning, 2000).

In addition to those microorganisms discussed above, a number of pathogenic vegetative microorganisms are considered to threaten the safety of cold-fill products, in particular members of the Enterobacteriaceae, specifically *E. coli* and *Salmonella*. These pathogens do not normally influence the safety of warm- and hot-fill products because the heat applied during cooking and filling rapidly inactivates these, and other, vegetative microorganisms. There are no specific

pathogen associations for warm-fill or hot-fill products, and the microbiological safety of these products is generally assumed to arise from application of good manufacturing practice (GMP) (Michels and Koning, 2000).

2.2.3 The CIMSCEE Code for microbiologically stable and safe cold-filled sauces, dressings and mayonnaises

The microbiological safety and stability of cold-filled acidic sauces and dressings is conferred predominantly by the addition of acetic acid. The commercial production of emulsified and non-emulsified sauces and dressings containing acetic acid is popularly guided by the Comité des Industries des Mayonnaises et Sauces Condimentaires de la Communauté Économique Européenne's Code (the CIMSCEE Code; CIMSCEE, 1992). The CIMSCEE Code recommends a hurdle technology approach to preservation, using a combination of aqueous phase acetic acid, sodium chloride, disaccharide (e.g. sucrose) and hexose (e.g. glucose) concentrations, and pH to preserve microbiological stability.

CIMSCEE defines an intrinsically microbiologically stable product as one in which $\Sigma > 63$, where:

$$\Sigma = 15.75 \times (1-\propto) \times (\text{total acetic acid\%}) + 3.08 \times (\text{NaCl\%}) + (\text{hexose\%}) + 0.5 \times (\text{disaccharide\%}) \quad \text{(Equation 1)}$$

and $(1-\propto)$ is the proportion of total acetic acid that is in the undissociated form, and other variables are as suggested by their names and with concentrations expressed as % wt/wt of aqueous phase (CIMSCEE, 1992*)*. Formulations with $\Sigma > 63$ are predicted to be microbiologically stable, "reducing numbers "(unspecified reduction) of the principal spoilage microorganisms over a 3 week period, and not allowing growth over a 6 week period.

The CIMSCEE Code advises that product formulations may still be microbiologically stable even if $\Sigma(s) \leq 63$. An example of this referred to in the Code is the use of other preservative hurdles, such as sorbic acid, the effects of which cannot be predicted by Equation 1. For such products, the Code recommends challenge testing to establish microbiological stability and safety.

Building upon its definition of an intrinsically microbiologically stable product, CIMSCEE defines an intrinsically microbiologically safe product as one in which $\Sigma s > 63$, where:

$$\Sigma s = 15.75 \times (1-\propto) \times (\text{total acetic acid\%}) + 3.08 \times (\text{NaCl\%}) + (\text{hexose\%}) + 0.5 \times (\text{disaccharide\%}) + 40 \times (4.0-\text{pH}) \quad \text{(Equation 2)}$$

and variables are as discussed for Equation 1. Formulations with $\Sigma s > 63$ are predicted to deliver a 3-$\log_{10}$ reduction of *Salmonella* or *E. coli* in < 72 h at 20°C (CIMSCEE, 1992), and are regarded as safe.

2.3 *E. coli* and *Salmonella* in cold-filled acid sauces

2.3.1 Introduction

Although published in 1992, the CIMSCEE code is underpinned by a set of experiments performed more than twenty years earlier by Tuynenberg Muys (1971), who compared the survival of single strains of *Salmonella* Bredeney and *Salmonella* Typhimurium with *Escherichia coli* and *Aerobacter aerogenes* to assess the safety of acetic acid containing dressings and sauces against the enterobacteriaceae, and to select a suitable non-pathogenic surrogate organism for use in challenge testing of sauce and dressing formulations.

When Tuynenberg Muys (1971) performed his experiments the pathogen of greatest concern for sauces and dressings containing acetic acid was *Salmonella*, because of the use of raw eggs in commercial manufacture at that time. As the use of pasteurised egg in commercial products is now nearly ubiquitous *Salmonella* is of less concern (Smittle, 2000) and use of the CIMSCEE safety score has in many cases come to be considered irrelevant, in favour of a focus on GMP and an integrated evaluation of raw material, processing and shelf-life requirements (Michels and Koning, 2000).

As concerns about the relevance of *Salmonella* in cold-fill products declined, concerns about the hazard of acid tolerant enterohaemorrhagic, or Shiga-toxin-producing *E. coli* (EHEC / STEC) grew. The first recognised outbreak of *E. coli* O157:H7 occurred in 1982 (Riley *et al.* 1983), and this pathogen has remained of concern ever since. STEC are highly virulent in humans at low doses, and cause serious acute illness and long-term sequelae (Bell, 2002). In about 5% of patients *E. coli* O157:H7 infection progresses to haemolytic-uremic syndrome, which may result in kidney failure and death (Mead and Griffin, 1998).

Outbreaks involving acidic foods including apple cider (Steele *et al.,* 1982; Besser *et al.,* 1983), dry, fermented sausage (CDCP 1995) and yoghurt (Morgan *et al.,* 1993) drew attention to the acid tolerance of *E. coli* O157:H7. Weagent *et al.* (1994) reported an outbreak of *E. coli* O157:H7 involving an estimated 300 people, and linked to commercial mayonnaise. However, this outbreak was later determined to be due to cross-contamination from uncooked meat (Michels and Koning, 2000). Overall, epidemiological evidence and likelihood-of-contamination assessments have concluded that commercially produced acidic dressings and sauces are not a high-risk vehicle for STEC (Erickson *et al.*, 1995).

Despite the above, concerns about the potential for these pathogens to contaminate such products remain. In particular, it has been acknowledged that there remains the potential for contamination or recontamination of pasteurised egg products (WHO / FAO, 2002; NSWFA, 2005; USDA, 2006). In New South Wales in Australia, licensed egg businesses are still required to analyse every 10th batch of pasteurised egg products for *Salmonella* (NSWFA, 2010). Further, ingredients used in the manufacture of cold-filled acid dressings and sauces, including herbs, spices and onion, are a potential source of pathogens. In one study of spices imported into Australia, *Salmonella enterica* was isolated from 1.5 and 8.2% of white and black peppercorns respectively (Pafumi, 1986). In 2010, the United States Food and Drug Administration announced a voluntary recall of, and the Canadian Food Inspection Agency issued a public warning against, a brand of pepper and a large number of pepper products potentially contaminated with *Salmonella* (US FDA, 2010; US FDA 2010b; CFIS, 2010). Low levels of faecal indicator

organisms have also been detected in onions; in a recent Australian survey (WAFMP, 2006) *E. coli* was detected in 2% of 55 whole onion samples tested.

An understanding of the ability of pathogenic *E. coli* and *Salmonella* to persist in contaminated acidic sauces and dressings is valuable for assessing the human health risks from food preparation and handling practices associated with cold-filled acid sauces, including re-use during open shelf-life, and use in the preparation of mayonnaise-based salads (and especially those containing meat). Such data is also potentially of value to manufacturers of acidic sauces and dressings (both large and boutique-scale), in updating the industry's understanding of the behaviour of *E. coli* and *Salmonella* in these products, and assessing the continued value of the CIMSCEE Code as an industry standard.

2.3.2 Inactivation of *E. coli* and *Salmonella* in cold-filled acid sauces, and similar

As discussed (Section 2.3.1), the microbiological safety predictions of the CIMSCEE code are underpinned by the date of Tuynenberg Muys (1971), comparing the survival of *Salmonella* Bredeney and *Salmonella* Typhimurium with *Escherichia coli* and *Aerobacter aerogenes*. The compositions tested by Tuynenberg Muys (1971) contained egg yolk, acetic acid, and salt, representing the water phase of a mayonnaise. Log_{10} reductions were determined after 48 and 72 h of incubation at 20°C. In this manner, it was determined that at least a 3-log_{10} reduction of Enterobacteriaceae could be achieved within 72 h at pH 4.1 with 2.14% acetic acid and 7.9% NaCl, but not at pH 4.21 with 2.15% acetic acid and 9.0% NaCl (Tuynenberg Muys, 1971). Therefore, the only modification to the CIMSCEE stability formula (*see* Equation 1, Section 2.2.3) to accommodate prediction of microbiological safety was the addition of the pH term (i.e. "40 x (4.0-pH)" (*see* Equation 2, Section 2.2.3) (CIMSCEE, 1992).

Beyond pH itself, pH and acetic acid are related in the CIMSCEE safety equation (Equation 2, *see* Section 2.2.3) by the multiplier "(1-$\propto$)", representing the proportion of total acetic acid that is in the undissociated form. As discussed (*see* Section 2.4.1), it is generally assumed that only the undissociated (or 'protonated') form of acetic acid is able to cross the cytoplasmic membrane

(Salmond *et al.*, 1984). This assumption is supported by the observation that, at a given acetic acid concentration, inactivation is increased as pH is decreased, and undissociated acetic acid thereby increased (Eklund, 1989). In a review of the microbiological safety of dressings and sauces manufactured in the USA (Smittle 2000), inactivation data for *Salmonella* spp. and *E. coli* O157:H7 from numerous published studies revealed pH to be statistically significant ($P < 0.05$) for both organisms. However, the effect of total acetic acid (measured as titratable acidity), was also significant for *E. coli* O157:H7, although not for *Salmonella* (Smittle 2000). It is noteworthy that although the CIMSCEE Code (CIMSCEE, 1992) recommends assessment of survival of *E. coli* for the determination of microbiological safety, *Salmonella* spp. and *E. coli* can differ in their response to acidic conditions. Tsai and Ingham (1997) individually inoculated four strains of *E. coli* and three strains of *Salmonella* into tomato ketchup acidified with acetic acid to pH 3.6 and compared inactivation at 23°C. After 6 h incubation the average inactivation was 1.4-$\log_{10}$ cfu/g for *E. coli*, while for the *Salmonella* the average inactivation was 3.6-$\log_{10}$ cfu/g over this time. *E. coli* (including STEC) are generally considered more acetic acid resistant, although more pH sensitive than *Salmonella* (Michels and Koning, 2000).

The order of effectiveness of organic acids against pathogenic bacteria is considered to be acetic, followed by lactic, then citric acids (Smittle, 2000). Unfortunately, most published studies that compare inactivation in the presence of different acids are not designed to distinguish the effects of organic acids because they confound acid concentration and pH, neglecting to determine the undissociated acid concentration.. However, when acetic acid was used to lower the pH of a buffered medium to pH 3.7, it took 4 h at 20°C for a 1-$\log_{10}$ inactivation of *E. coli* to occur compared with 22 h when a non inhibitory acid (i.e. gluconic acid) was used (Breidt et al. 2004). In another study, equimolar concentrations (200 mM) of citric or acetic acids, adjusted to pH 4.5 with HCl and resulting in 1.2% total acetic acid or 0.38% citric acid, revealed much greater inactivation by acetic acid of a four strain cocktail of *Salmonella enteritidis* PT4 (Alexandrou et al. 1995). While it took seven days for cells to reach undetectable levels with acetic acid (> 6-$\log_{10}$ inactivation), after nine days there was only a 2-$\log_{10}$ inactivation in broths containing citric acid (Alexandrou *et al.,* 1995).

Also noteworthy is that while the CIMSCEE Code (CIMSCEE, 1992) stipulates pre-adaption of spoilage microorganisms prior to challenge testing, there is no such stipulation for pathogen testing, reflecting the heritage of the studies (Tuynenberg Muys, 1971) on which the CIMSCEE Code is based. However, many studies have identified the importance of pre-adapting *E. coli* (Leyer *et al.,* 1995; Ryu *et al.,* 1999*)* and *Salmonella* (Samelis *et al.,* 2003) isolates prior to assessment of their acid tolerance. For example, pre-adapted *Salmonella* Typhimurium cells challenged in a pH 3.7 broth containing 2.9% total acetic acid were inactivated by only 1-$\log_{10}$ after 2 h, whereas non-adapted cells were not detectable (>4-$\log_{10}$ inactivation) at the end of the same time period (Samelis *et al.,* 2003). Statistically significant variation in acid resistance among strains of *E. coli* (Large *et al.,* 2005) and *Salmonella* (Berk *et al.,* 2005) have also been reported. For *E. coli* O157:H7, 92% of the variation in survival after 6 h in pH 2.0 broth was reported (Large et al., 2005). Among thirty-seven *Salmonella* Typhimurium serovar DT104 strains, a significant difference amongst the strains ability to survive exposure to pH 2.5 for 2 h was observed with survival ranging from 0.0001 to 36% (Berk et al., 2005).

The form of Equation 2 (*see* Section 2.2.3) embodies the concept that increasing concentrations of osmolytes (NaCl, disaccharides, hexoses) will reduce pathogen survival in cold-filled acetic acid-containing products. Until recently, there has been little specific investigation into the effect on pathogen inactivation of simultaneous exposure to acetic acid and osmolytes: several more recent publications have explored antagonistic effects of these hurdles, and these are reviewed in Section 2.3.3, below. Yamamoto et al (1984) reported that sodium chloride increased the antimicrobial action of acetic acid, and Rowbury *et al.* (1994) reported that *E. coli* incubated at elevated NaCl concentrations exhibited a marked sensitivity to a subsequent acetic acid challenge. Acetic acid in the form of vinegar and NaCl was also reported to have a synergistic inhibitory effect on *E. coli* O157:H7 (Entani *et al.*, 1997).

An important factor not included in Equation 2 is incubation, or storage, temperature. Numerous studies have observed that pathogenic microorganisms are able to survive longer in inimical environments under refrigeration compared to ambient temperatures (Eribo and Ashenafi 2003; Hathcox *et al.,* 1995; Lindqvist and Lindblad 2009; Lock and Board 1994; Lock and Board 1996;

Perales and Garcia 1990; Roller and Covill 2000; Ross *et al.,* 2008; Tsai and Ingham 1997; Weagent *et al.,* 1994; Zhao and Doyle 1994). Weagant *et al.* (1994), inoculated 7- to 8-$\log_{10}$ cfu/g of *E. coli* O157:H7 into pH 3.7 mayonnaise and found that after 72 h incubation at 25°C there was at least 6-$\log_{10}$ cfu/g inactivation, while it took 35 days for a 5-$\log_{10}$ inactivation to occur at 7°C. *Salmonella enteritidis* PT4 cells inoculated into pH 4 mayonnaise were inactivated by 6-$\log_{10}$ cfu/g after 72 h at 24°C but there was a less than 1-$\log_{10}$ inactivation when the mayonnaise was stored at 4°C over this time (Perales and Garcia, 1990). Roller and Covill (2000) compared inactivation at 5 and 25°C for *S. enteritidis* PT4 in pH 4.4 mayonnaise. After 48 h incubation at 25°C there had been 5-$\log_{10}$ inactivation while at 5°C there had only been 3-$\log_{10}$ inactivation at the completion of the 192 h experiment.

Perhaps the most comprehensive study of pathogen inactivation in a mayonnaise model system is McKellar et al. (2002) who developed a probability model describing the interface between survival and death (P = 0.5 (50% probability of survival)) of *E. coli* O157:H7 for factors of temperature (10 - 30°C), NaCl (0.5 – 16.5%), pH (3.5 – 6.0), acetic acid (0 – 4%) and sucrose (0 – 8%). A logistic polynomial regression was developed, with a total of 1820 factor combinations, with undissociated acetic acid replacing total acetic acid in the model. The model successfully predicted 72 h survival or death in 1772 (97.4%) of samples, and was correct for 24 / 30 (80%) of mayonnaise samples used in validation. The 72 h survival / death interface for pH as influenced by acetic acid was at pH ≤ 3.5 for acetic acid ≤0.5%, increasing to pH 4.0 – 4.5 at 1.2% acetic acid, and pH 4.5 – 5.0 at 3 and 4% acetic acid. The 72 h survival / death interface for pH as influenced by temperature (with NaCl at 0.5% and acetic acid at 3%) was between pH 3.5 – 4.0 for 10 to 20°C, increasing to pH 4.5 at 30°C. While the transition between 10% probability (P=0.1) of survival and 90% probability (P=0.9) of survival was abrupt over the whole temperature range, the transition with acetic acid as the variable was broader at lower as compared to higher acetic acid concentrations (McKellar et al. 2002).

2.3.3 Protection of *E. coli* and *Salmonella* in cold-filled acid sauces, and similar

McKellar *et al.* (2002) also explored the effect of NaCl concentration on the pH interface at 3% acetic acid and 30°C, and found this to be at pH 4.5 – 5.0 at 0.5% NaCl, reducing to pH 4.0 – 4.5 at ≥1.0% NaCl. The interface between P=0.1 and P=0.9 was abrupt over the entire range of NaCl concentrations. Simulations with 2 - 4% acetic acid and increasing levels of NaCl or sucrose showed a generally increasing probability of survival up to ~3.5 - 4.5% NaCl and 4 - 8% sucrose, especially at higher temperatures and lower acetic acid concentrations. Additionally, upon comparing their observed results with CIMSCEE predictions, the authors noted that CIMSCEE gave 'fail-dangerous' predictions (*i.e.* false negatives, predicting death when survival occurred) when the concentration of NaCl and / or sucrose in their mayonnaise models exceeded 2.5 and / or 4% respectively (McKellar *et al.* 2002).

Studies prior to McKellar et al. (2002) suggested that *Salmonella* may be protected by the addition of NaCl during inactivation under acidic conditions. Larson et al. (1993) showed that the rate of inactivation of *Salmonella* Heidelberg was increased in unsalted rennet whey at pH 4.8 or 5.6, compared with in rennet whey salted at 3.6 or 4.8% NaCl-in-moisture. Similarly, Radford and Board (1995) describe unpublished work that found that small concentrations of NaCl slowed the inactivation rate of *Salmonella* Enteritidis in mayonnaise.

Other studies conducted under growth-permissive, but acidic, conditions have shown that growth of both *Salmonella* and *E. coli* may be stimulated by the addition of NaCl. In nutrient broth at pH 5.5, Radford and Board (1995) demonstrated that *Salmonella* Enteritidis PT4 and PT6 (but not PT8) were stimulated by the presence of around 2% (w/v) NaCl or KCl in the presence of acetic acid, but not citric, propionic or hydrochloric acids. No stimulation was observed for sucrose or glycerol at concentrations ranging from 2 to 8% (w/v) (Radford and Board, 1995). Similarly, Jordan and Davies (2001) showed that 4 to 6% (w/w) NaCl stimulated growth of *E. coli* in tryptone phosphate broth at pH 5.0 in the presence of lactic acid.

In a study focussed on the microbiological safety of fermented sausages, Casey and Condon (2002) showed that the addition of 4% NaCl improved survival of an enteropathogenic *E. coli* (EPEC) strain O157:H45 (as a model for enterohaemorrhagic *E. coli* (EHEC)) in tryptic soya broth acidified to pH 4.2 with lactic acid. It was noted that addition of 4% NaCl after acid exposure did not reverse acid damage, and so it was suggested that NaCl must instead act to prevent the occurrence of acid damage. The cytoplasmic pH of *E. coli* challenged at pH 4.2 with lactic acid was found to be pH 5.23 in the absence of NaCl, and pH 5.79 in the presence of 4% NaCl. *E. coli* cell volume decreased from 3.31 to 1.43 µL/mg protein in the presence of 4% NaCl. The authors also showed that the addition of fructose, Na_2SO_4 or KCl in place of NaCl improved the *E. coli* survival rate, but not to the same extent as that of NaCl (Casey and Condon, 2002).

Casey and Condon (2002) also noted that the addition of 4% NaCl appeared to have an even more pronounced effect on pathogen survival in TSB acidified to pH 4.2 with acetic acid. Lee and Kang (2009) showed that the addition of 3% NaCl to Luria-Bertani broth significantly improved resistance of five strains of *E. coli* O157:H7,to inactivation with 0.25% acetic acid. As observed by Casey and Condon (2002), Lee and Kang (2009) citing their unpublished data, showed that addition of 3% NaCl post-acid challenge did not improve survival of *E. coli* O157:H7, and therefore appeared unable to reverse acid damage. In a follow-up study (Lee et al., 2010) the antagonistic effect of 3% NaCl on inactivation of *E. coli* O157:H7 in the presence of acetic acid in pickled cucumber was confirmed. Greater than a 5-$\log_{10}$ reduction in *E. coli* numbers was observed at 0.5 and 0.75% acetic acid in 50 and 94% pureed cucumber products, but for these same products formulated with 3% NaCl, greater than 5-$\log_{10}$ reductions occurred only in the presence of 2% acetic acid (Lee et al. 2010).

2.3.4 Proposed mechanisms of osmolyte protection against inactivation of *E. coli* by acetic and other acids

Casey and Condon (2002) hypothesised the protective effect of NaCl and other solutes on acid inactivation of *E. coli* O157:H45 to be partially osmotic in nature, but principally due to the increase in cytoplasmic pH they observed in the presence of NaCl. They suggested it was unlikely

that NaCl could prevent uptake of protons into the *E. coli* cytoplasm, and instead speculated on the possible existence of a sodium-proton antiporter system operating in an opposite fashion to the analogous NhaA system (Casey and Condon, 2002). The NhaA sodium-proton antiporter functions to decrease the intracellular pH of *E. coli* at alkaline pH by importing H^+ in exchange for exporting Na^+ (Sakuma *et al.*, 1984). At acidic pH, an analogous system could import Na^+ in exchange for exporting H^+ (Casey and Condon, 2002).

More recently Kitko *et al.* (2010), by determining cytoplasmic pH using GFPmut3 fluorimetry, have confirmed that a number of osmolytes contribute to cytoplasmic pH homeostasis in *E. coli* K-12, improving the ability of the bacterium to recovery from rapid acid shift imposed by HCl. Recovery of cytoplasmic pH was equally achieved by supplementation with NaCl, KCl, proline and sucrose (Kitko *et al.*, 2010). While the mechanism of the link between osmoregulation and pH homeostasis was not able to be concluded by the authors, it was proposed that it may involve maintenance of cell volume, thereby avoiding concentration of acid species and a decrease in pH (Kitko *et al.*, 2010). However, Casey and Condon (2001) showed that a decrease in cell volume correlated with an increase in intracellular pH in the presence of lactic acid, and suggested that this may be due to an increase in buffering capacity by concentration of other (i.e. anionic) cytoplasmic constituents. High anion pools are already understood to be of benefit in maintaining cell turgor, since these pools allow for the accumulation of K+ ions (Booth, 1999). It is possible that different effects may predominate depending on the extent of cell volume changes, as well as on the type of and concentration of the acidulent, and the concentration of the osmolyte, since all of these factors vary between the Kitko *et al.* (2010) and Casey and Condon (2001) studies.

In another, recent study modelling the effects of NaCl, acetic acid and intracellular pH on survival of *E. coli* O157:H7, a protective effect of NaCl concentration at selected acetic acid concentrations was observed (Hosein et al., 2011). At pH 3.2, with undissociated acid concentrations of 20 mM or less, long term survival (between 50 and 100 h) was better with 4% NaCl that with 2% NaCl. At higher undissociated acetic acid concentrations, survival decreased as NaCl and undissociated acetic acid concentrations increased. At 4% NaCl and less than 20 mM acetic acid, improved survival of *E. coli* was correlated with a lower intracellular pH,

measured after 30 min exposure to acid conditions. Hosein *et al.* (2011) suggested that the lower intracellular pH could serve to reduce accumulation of the acetate anion, which has been proposed as a mechanism of cell death (Russel, 1992; Diez-Gonzalez and Russell, 1997). At 2% NaCl, a lower intracellular pH correlated with a decrease in the observed 5-$\log_{10}$ reduction time, suggesting that survival was reduced with lower intracellular pH. Based on the undissociated acid concentration and calculated from the intracellular pH data, increasing the intracellular acetate anion concentration was also found to correlate with a decrease in survival. However, at 4% NaCl and ≤10 mM undissociated acetic acid, the intracellular pH increased, prolonging *E. coli* survival. However, the intracellular pH with 4% NaCl and 5 mM undissociated acetic acid was similar to the intracellular pH with 2% NaCl and 40 mM undissociated acid (around pH 5.8), yet the population exposed to 2% NaCl and 40 mM undissociated acid died were much more rapidly inactivated. The authors concluded that the relationships among the intracellular pH, acetic acid anion concentration, and cell survival remained unclear, and postulated that other factors, including the proton motive force (ΔP) may also help elucidate the mechanism of osmolyte protection against acetic acid inactivation (Hosein *et al.* 2011).

Finally, a possible role for Na^+ in acid resistance has been linked to induction of the Gad regulon (*gadA*, *gadBC*), which regulates glutamate-dependent acid resistance (Richard and Foster, 2007). The regulator protein GadE is the essential activator of the Gad regulon, and the transcription of this protein is itself controlled by a number of proteins, including GadX and GadW, whose sensory signals are unknown. Decreasing Na^+ was found to coincide with decreased resistance to acidic conditions simulating those of gastric transit, together with decreased expression of *gadE*, *gadA* and *gadBC*, except in the presence of GadX and GadW. As Na^+ levels did not regulate *gadX* and *gadW* transcription, it has been proposed that GadX and GadW are possible candidates for intracellular Na+ concentration sensors (Richard and Foster, 2007).

2.4 Mechanisms of acetic acid inactivation and resistance

2.4.1 Introduction

Acetic acid (CH_3COOH) is a short chain fatty acid used extensively in food preservation (Cherrington *et al.*, 1991). Acetic and similar organic acids are often referred to as weak acids, since they do not readily donate protons in aqueous solution, and so remain partially undissociated, dependent on pH. Dissociation increases with increasing pH. The pKa indicates the pH at which 50% of the acid will be present in the dissociated form and 50% in the undissociated form. The pKa for acetic acid is approximately 4.8 (Cherrington *et al.*, 1991).

The mode of transport of short chain fatty acids (including acetic acid) into microbial cells is generally assumed to be by passive diffusion of the undissociated (lipophilic) form across the cytoplasmic membrane (Salmond *et al.*, 1984). For some microorganisms, there is evidence also that energy-linked carriers and membrane potential ($\Delta\psi$) may play a role in the transport of short chain fatty acids (Cherrington *et al.*, 1991). *E. coli* and *Salmonella* can actively transport acetic acid into the cell and utilise it as a carbon source (Nunn, 1986). Despite this, resistance of Gram-negative cells to the antimicrobial effects of acetic acid has been postulated to be mediated by the lipopolysaccharide layer of the outer membrane, by preventing acid entry into the cell (Sheu and Freese, 1973).

Several cellular components and processes have been proposed as targets for the action of organic acids, including acetic acid. These include intracellular acidification and anion accumulation affecting enzymes, macromolecular synthesis, and DNA, and cell membrane integrity and function (Cherrington *et al.*, 1991), and energy diversion and uncoupling (Freese *et al.*, 1973; Sheu *et al.*, 1975; Baronfsky *et al.*, 1984, Salmond *et al.*, 1984).

2.4.2 Intracellular acidification and pH homeostasis

When a lipid-permeable weak acid such as acetic acid is added to a cell with a pre-existing transmembrane pH gradient (i.e. ΔpH, alkaline inside), equilibration of the undissociated weak acid across the cytoplasmic membrane will result, with subsequent dissociation of the weak acid in the cytoplasm in accordance with the intracellular pH (Booth and Kroll, 1989; Lambert and Stratford, 1999).

Conceptually, intracellular acidification, and a concomitant decrease in the activity and molecular stability of macromolecules including enzymes, other proteins, and nucleic acid materials appears the most straightforward explanation for the detrimental effect of low pH in general, including acetic acid as an effector of low pH, on microbial growth (Booth and Kroll, 1989; Small *et al.*, 1994; Hall *et al.*, 1995). Enzyme activity is generally lowered by acid pH values (Cherrington *et al.*, 1991). A DNA polymerase deficient mutant of *E. coli* has been shown to be more sensitive than its isogenic parent in the presence of acetic acid at pH 3.5 (Sinha, 1986), and it has been postulated that acid anions may interfere with DNA molecule conformation by interacting with ion charges around it (Cherrington *et al.*, 1991).

Since the structural and functional properties of cellular macromolecules are maintained over only a relatively narrow pH range, the ability to maintain consistent intracellular pH, termed pH homeostasis, is generally considered vital for microbial growth (Booth, 1985). Survival of *E. coli* and *Salmonella* under acidic conditions has therefore been explained mainly by the existence of a variety of acid resistance systems that help to maintain a high intracellular pH (Foster and Hall, 1991; Warnecke and Gill, 1995). Over an external pH range of pH 4.5 to 7.9 the intracellular pH of *E. coli* has been shown to vary by less than 0.1 unit per unit change in extracellular pH (Slonczewski *et al.*, 1981; Hill *et al.*, 1995; Slonczewski and Blankenhorn, 1999). Further, measurements of the intracellular pH of unadapted versus adapted cells at an external pH of 3.3 indicate that the intracellular pH of unadapted cells drops 0.5 to 0.9 pH unit lower than that of the adapted cells (Foster and Hall, 1991). In contrast, however, the intracellular pH of *E. coli* O157:H7

can fall during exposure to acetic acid (Diez-Gonzalez and Russell, 1997b) the implications of which are discussed in Section 2.4.4, below.

Intracellular pH homeostasis is effected by both constitutively expressed and inducible regulatory systems. It is generally considered that constitutively expressed systems are of most relevance when extracellular pH is within one pH unit of the optimum pH for growth of the microorganism (Slonczewski and Foster, 1996), and that housekeeping pH homeostasis mechanisms are sufficient to maintain intracellular pH when the extracellular pH is above pH 4 (Foster, 1999). Among the constitutively expressed systems for pH homeostasis are what are regarded as passive and active systems (Booth, 1985). The buffering capacity of the cytoplasm and the cytoplasmic membrane itself are the most well-recognised of the passive constitutively expressed systems contributing to pH homeostasis (Booth and Kroll, 1989). Active constitutively expressed systems include a variety of membrane-bound proton / cation antiporter systems, including K+/H+ and Na+/H+ antiporters (Booth, 1985; Foster, 1993), cytochrome oxidase D and O, and the F_1F_o ATPase (Cotter *et al.*, 1990; Kasimoglu *et al.*, 1996; Foster and Hall, 1991).

Under more extreme extracellular pH (i.e. pH < 4) additional pH homeostatic and low pH repair systems may be induced as part of the acid tolerance response (ATR), resulting in the up and down regulation of synthesis of a number of proteins (Foster, 1999). Among those inducible proteins believed to contribute directly to pH homeostasis are the membrane porins OmpC and OmpF (Heyde and Portalier, 1987; Thomas and Booth, 1992), and a variety of decarboxylases (lysine, arginine, glutamate, ornithine, tyrosine and histidine) (Gale, 1946) that function with membrane bound antiporters to take up amino acids and efflux the polyamines resulting from intracellular protonation (Gale and Epps, 1942; Meng and Bennet, 1992).

Compared with our knowledge of the importance of pH homeostasis under growth permissive conditions (as above), the importance of pH homeostasis in microbial survival under non-growth permissive conditions, is much less clear. Under growth permissive conditions, the current conceptual model holds that a change in extracellular pH results in a transient perturbation, followed by restoration, of intracellular pH prior to resumption of growth (i.e. cellular division)

(Slonczewski *et al.*, 1981; Zilberstein *et al.*, 1982; Salmond *et al.*, 1984; Zilberstein *et al.*, 1984). In terms of population kinetics under growth permissive conditions, the time taken to restore intracellular pH is manifested as a lag phase, the duration of which is dependent both on the magnitude of the transient intracellular pH change, as well as the rate of restoration of intracellular pH (Zilberstein *et al.*, 1984). Under conditions that are in any case non-growth permissive, it is worthwhile considering whether the restoration of intracellular pH remains as crucial to cell survival as to growth.

Jordan *et al.* (1999) examined the survival of a nontoxigenic *E. coli* O157:H7 under non-growth permissive conditions at pH 3 achieved using hydrochloric acid (HCl). They found that even after exposure for three days at pH 3, significant numbers of survivors could be detected from inoculated populations of exponential, stationary, and acid-adapted (pH 5.0 for 1h prior to challenge) exponential phase cells, but the survival curves for each of the three inoculated populations were significantly different. Using proton flux measurements, they determined that the initial rates of viability loss of the three inoculated population types were well correlated with net proton accumulation; that is, the more resistant populations accumulated protons more slowly, with the protection conferred by acid adaptation appearing greater than that afforded by stationary phase physiology. Using two-dimensional polyacrylamide gel electrophoresis, the authors went on to examine and compare the protein composition of the cell envelope of the three inoculated populations, which they determined to be significantly different. Based on their observations of membrane protein differences among the three inoculated populations, the authors speculated that such membrane protein changes might either result in a decreased permeability of protons into the cell (for example, via the outer membrane porin, PhoE), or an enhanced ability to efflux protons from the cell. Regardless, Jordan *et al.* (1999) concluded that the ability to maintain intracellular pH was a defining characteristic for long term survival of *E. coli* under non-growth permissive conditions.

2.4.3 Energy diversion and uncoupling

Intracellular pH homeostasis is generally considered to be an energy requiring process (Kroll and Booth, 1981; Booth, 1985). The lowering of pH is opposed by removal of excess protons at the expense of adenosine triphosphate (ATP) (Booth, 1985). If microorganisms experiencing acid stress divert energy into pH homeostasis, maintenance energy demands can eventually exceed the cell's energy producing capability, resulting in inactivation (Leistner, 2000).

In respiring bacteria under physiological conditions, the F_1F_o ATPase generates ATP using ΔP (the proton motive force) created by the electron transport chain as a source of energy, in the process of oxidative phosphorylation (Mitchell, 1961). In this process, protons flow down the proton gradient (ΔP) across the cytoplasmic membrane through the F_1F_o ATPase. This proton flow forces the rotation of a part of the F_1F_o ATPase, which is in effect a rotary mechanical motor. The rotation allows the phosphorylation of adenosine diphosphate (ADP) with P_i to form ATP (Nakamoto *et al.*, 2000). Thus electrochemical energy contained in the ΔP is converted into mechanical energy in form of subunit rotation, and back into chemical energy as ATP (Nakamoto *et al.*, 2000).

The F_1F_O ATPase is a reversible enzyme (Nakamoto *et al.*, 2000). The electrochemical energy stored in the ΔP is energetically equivalent to chemical energy in the phosphate bond of ATP, and both energy forms are interconvertable, as described by chemiosmotic theory (Mitchell, 1961). As discussed, in ATP synthesis mode the enzyme makes ATP from ADP + P_i using the energy of proton electrochemical potential difference. In the reverse mode the energy of ATP hydrolysis is used to pump protons back across the cytoplasmic membrane, creating potential energy in the form of a pH gradient and an electrical potential gradient. Thus in fermenting microorganisms the F_1F_O ATPase functions as an ATP-driven proton pump. In *E. coli* and *Salmonella* both functions are therefore important, depending on the cell environment. It has been proposed that under acidic conditions, the energy required to rid the cytoplasm of protons via reverse running of the F_1F_O ATPase drains the cell of ATP, resulting in death (Baronfsky, 1984; Axe and Bailey, 1995). However, it should be noted that under acidic fermentation conditions, it has been suggested that

the hydrogenases Hyd-1 and Hyd-2 may be of greater importance to H^+ efflux in *E. coli* than is the F_1F_O ATPase (Blbulyan *et al.*, 2011).

The ability of weak organic acids such as acetic acid to dissipate ΔP (i.e. neutralise the electrochemical gradient) has been held to parallel that of synthetic uncouplers such as carbonylcyanide-m-chlorophenylhydrazone (CCCP) (Freese *et al.*, 1973; Sheu *et al.*, 1975; Baronfsky *et al.*, 1984, Salmond *et al.*, 1984). As mentioned, ΔP is comprised of two components, Δψ (the membrane potential) and ΔpH (the transmembrane pH gradient). The Δψ is caused by a charge difference across the cytoplasmic membrane (when the protons move without a counter-ion), and ΔpH is caused by a differential concentration of hydrogen (more correctly, hydronium) ions across the cytoplasmic membrane. The combination of these two factors determines the thermodynamically favourable direction for an ion's movement across a membrane. Bacteria normally maintain a Δψ of over 100 mV across the cytoplasmic membrane, with the interior side negative (i.e., by convention, -100mV). A 10-fold gradient in the $[H^+]$ has recently been shown to be energetically equivalent to 102mV of Δψ (Krajl *et al.*, 2012), thus where there is a ΔpH around three pH units (e.g. intracellular pH ~ 7 and extracellular pH ~ 4) the ΔP is around −400mV.

Synthetic uncouplers are extremely effective proton translocators and even very small amounts are often able to decrease both ΔpH and Δψ (Russell, 1992). The undissociated species travels across the cytoplasmic membrane and dissociates in accordance with the ΔpH. The anion remains associated with the membrane, and is driven by the Δψ to the external surface of the cytoplasmic membrane, where it is again protonated. Uncoupling thus requires a high degree of lipid permeability of both the undissociated species and of the anion (Russell, 1992).

Short chain fatty acids are only weakly lipophilic, since in fatty acids with only a small number of carbon atoms, the polar acid functional group dominates over the non-polar character of the (very short) hydrocarbon chain (Steiner and Sauer, 2001). The polar anionic species are lipophobic, and thus it has been argued that these should not freely traverse the cytoplasmic membrane (Russell, 1992). A weak acid which has no significant permeability of the anion will only dissipate the ΔpH, and usually this leads to an increase in the Δψ (Russell, 1992). For instance, Baronofsky

et al (1984) noted that acetate caused some decrease in the ΔpH of *Clostridium thermoaceticum*, but this decrease was partially compensated by an increase in Δψ. Russell (1992) has held that there is in fact little evidence that even high concentrations of fermentation acids can translocate protons at a rate fast enough to dissipate the ΔP (Russell, 1992). However, Axe and Bailey (1995) in comparing measured concentration ratios to those expected at equilibrium for various transport modes have countered that acetate is in fact a classical uncoupling agent, permeating the membrane at comparable rates in the dissociated and undissociated forms.

2.4.4 Cytoplasmic accumulation of acetate

As previously discussed, there are conflicting reports of pH homeostasis (Booth, 1985) and intracellular pH lowering (Diez-Gonzalez and Russell, 1997b) that leaves unclear the question of how intracellular pH and cell survival are related (Hosein *et al.,* 2011). While maintaining a high pH is likely to optimise metabolic enzyme function, allowing intracellular pH to fall may also be of benefit in reducing acetate anion accumulation (Russell, 1992; Diez-Gonzalez and Russell, 1997). Anions will accumulate in the cell cytoplasm in accordance with the Henderson-Hasselbalch equation (Russell, 1992). By allowing intracellular pH to fall, and thus maintaining a constant ΔpH, the cytoplasmic anion concentration will remain constant, irrespective of extracellular pH (Russell, 1992).

An excess amount of acetate anion in the cytoplasm may disturb physiological processes including the osmolarity of the cell (Barua *et al.*, 2002), resulting in macromolecular (Russell, 1992) and metabolic (Barua *et al.*, 2002) disruption. However, direct demonstration of anion toxicity has been confounded by the inability of dead cells to accumulate anions (Russell, 1992).

2.4.5 Membrane integrity and function

In addition to their potential to interfere with energy-linked functions (*see* Section 2.4.3), it is feasible that weak acids may also affect the integrity, and therefore the function of cell

membranes (Cherrington *et al.*, 1991). However, comparatively little research has been undertaken on the effects of weak acids, including acetic acid on cell membrane integrity.

With respect to the outer membrane of Gram negative bacteria, suspension of cells in solutions of extreme pH may result in irreversible surface disorganisation (James, 1991). Acidification has been used as the basis for extraction of outer membrane components from *E. coli* (Wolf-Watz *et al.*, 1973). It has also been demonstrated that some organic acids such as lactic acid are able to effect significant damage to the outer membrane of Gram-negative microorganisms (Alakomi *et al.*, 2000). It was initially supposed that, since LPS are non-covalently cross-linked and held in position at the outer membrane surface by divalent cations (Denyer & Maillard, 2002), chelation of divalent cations by the dissociated organic acid molecule might explain this damage. However, the same pattern of damage was also observed for fully dissociated mineral acids (HCl) (Alakomi *et al.*, 2000). It is now thought that the mechanism of damage to the outer membrane is by the protonation of anionic components (carboxyl and phosphate groups) of the LPS, thus weakening molecular interactions between the outer membrane components (Alakomi *et al.*, 2000). Yet it has been reported that acetic acid does not disrupt the outer membrane sufficiently to potentiate the effect of nisin (Helander and Mattila-Sandholm, 2000), and exposure to acetate buffer at pH 4.2 did not cause protein leakage in *E. coli* (Przybylski and Witter, 1979). However, exposure to acetate buffer at pH 3.2 – 3.5 resulted in some protein leakage for *Salmonella* (Blankenship, 1981).

There are few published studies that address the effect of acetic acid on cytoplasmic membrane integrity and function of *E. coli* or *Salmonella*. While it has been proposed that perturbation of cytoplasmic membrane function may be the primary cause of inhibition by more lipophilic acids (Freese *et al.*, 1973), such as sorbic acid (Stratford and Anslow, 1988), this mechanism of action is not generally considered important for acetic acid, owing to its greater polarity, and therefore decreased likelihood of remaining associated with the membrane (Eklund, 1973).

2.5 The cell envelope of *E. coli* and *Salmonella*

2.5.1 The outer membrane

Like the cytoplasmic membrane, the outer membrane of Gram negative bacteria is usually conceived as a lipid bilayer (*see* Section 2.5.3). Particularly in the case of chemical preservation strategies, the outer membrane, and more specifically the outer leaflet of the outer membrane, is the first part of a bacterial cell to experience a change in the cell's physicochemical environment (van der Mei *et al.*, 2000). In wild-type *E. coli* and *Salmonella* the outer membrane is a highly asymmetrical construction possessing limited regions of phospholipid bilayer; the outer leaflet of the outer membrane is primarily composed of lipopolysaccharide (LPS) molecules and proteins (Nikaido, 1979). LPS are in turn composed of a lipid section, lipid A (internal), a short oligosaccharide, and an O antigen that may be a long polysaccharide (external) (Nikaido, 2003), thus providing Gram-negative organisms with a protective hydrophilic outer surface characterised by a net anionic charge (Helander & Mattila-Sandholm, 2000). The major function of the outer membrane then is to act as a coarse filter excluding, in particular, large and hydrophobic (lipophilic) molecules (Nikaido, 1979).

The low permeability of the outer membrane of wild-type *E. coli* and *Salmonella* improves the resistance of these bacteria to bile salts and long-chain fatty acids that are encountered in their normal environment of the gastrointestinal tract (Nikaido, 1979). However, the diffusion of small hydrophobic molecules is greatly affected by the prevailing structure of LPS (e.g. wild-type versus deep-rough mutant) (Nikaido, 1993). The hydrocarbon chains of LPS are tightly clustered together with at least six chains connected to a single backbone structure and an absence of unsaturated fatty acids that reduces fluidity (Nikaido, 2003) and dramatically slows permeation in wild-type *E. coli* and *Salmonella* (Nikaido, 1979). In deep-rough mutants however, it is thought that the altered structure of LPS hinders the interaction of LPS and proteins in the outer leaflet, resulting in a decreased incorporation of outer membrane proteins, and in turn an increase in the area of phospholipid bilayer structure with greater permeability to small hydrophobic molecules (Nikaido, 1979). For example, the relative efficiency of hydrophobic inhibitors including novobiocin, phenol

and crystal violet was improved by ≥ 10 times in deep-rough mutants compared with wild-type cells (Nikaido, 1976).

In contrast to hydrophobic molecules, small hydrophilic molecules cross the outer membrane by non-specific diffusion through porin proteins (Nikaido, 1979). In these cases, the diameter of the porin is close to that of the diffusing solute, with water in the porin channel likely to be more strongly hydrogen-bonded and structured than the bulk water (Nikaido, 1979). Passive diffusion through porin channels becomes limiting if the size of the molecule is large, and in this (and other) cases, specific transport may occur through dedicated protein channels (Nikaido, 1979).

2.5.2 The periplasm and cell wall

The outer and cytoplasmic membranes of Gram-negative bacteria are separated by the periplasm, which represents around 20 – 40% of the cell volume (Stock *et al.,* 1977). The periplasm has been traditionally viewed as an intervening space between the membranes containing a thin peptidoglycan cell wall 20 – 30 Å (2 – 3 nm) in width (Kellenberger and Ryter, 1958). The cell wall has been described as an elastic mesh composed of glycan chains cross-linked by peptides (Koch and Woeste, 1992), connected to the outer membrane by Braun's lipoproteins (Braun and Rehn, 1969). The exact nature (if any) of the mechanical relationship between the cytoplasmic membrane and the cell wall of Gram negative bacteria remains unresolved (Sochacki *et al.*, 2011); it has been proposed that there may be physical anchor points (Bayer, 1968), or at least protein complexes that span the periplasmic space (Nikaido, 2003).

An alternate model of the periplasm is that of the periplasmic gel (Hobot *et al.* 1984). In this model, peptidoglycan fills the entire space between the cytoplasmic and outer membranes in the form of a well-hydrated gel around 13 nm thick. It is proposed that the gel has increased cross-linking in the section underlying the outer membrane, with less cross-linking towards the cytoplasmic membrane. This model dismisses as artefacts of sample preparation, concepts of adhesion and contact points between the cytoplasmic and outer membranes (Hobot *et al.*, 1984).

2.5.3 The cytoplasmic membrane

Biological membranes are composed of a mixture of phospholipids, proteins, and carbohydrates (Singer and Nicolson, 1972). Phospholipids consist of a hydrophilic and a hydrophobic moiety, are water insoluble and, in aqueous environments, form closed bimolecular sheets, which are referred to as lipid bilayers (Singer and Nicolson, 1972). Proteins within a lipid bilayer act in a number of capacities, including as receptors, pumps, enzymes and gates (Tan *et al.*, 2008). The proteins and phospholipids present in a bacterial membrane are held together by many cooperative, noncovalent interactions (Singer and Nicolson, 1972). Thus under physiological conditions bacterial membranes are fluid structures that can be thought of as two-dimensional solutions of oriented proteins and phospholipids, with phospholipids in particular diffusing rapidly within the plane of the membrane (Singer and Nicholson, 1972).

The relative percentages of the three major cytoplasmic membrane phospholipid headgroups in exponential phase *E. coli* cells are typically reported as 75% phosphatidylethanolamine, 20% phosphatidylglycerol, and 5% di-phosphatidylglycerol, commonly known as cardiolipin (Cronan, 2003). The phospholipid composition of *Salmonella* has been shown to be quite similar to that of *E. coli*, albeit with slightly decreased levels of anionic phospholipids, including cardiolipin, as a percentage of total phospholipids (Ames, 1968). It is known, however, that cardiolipin synthesis in *E. coli* increases dramatically during stationary phase (Hiraoka *et al.*, 1993). It has also been shown that cardiolipin-rich cytoplasmic membrane domains are predominantly located at the polar and septal regions of *E. coli* cells (Mileykovskaya and Dowhan, 2000). *E. coli* possesses three major fatty acids; palmitic (hexadecanoic) acid which is a saturated fatty acid, and palmitoleic (*cis*-9-hexadecanoic) acid and *cis*-vaccenic (*cis*-11-octadecanoic) acid, both of which are unsaturated fatty acids (Cronan and Rock, 1996). Other fatty acids, such as myristic acid are typically found in smaller proportions (Marr and Ingraham, 1962). In addition, and particularly during entry into stationary phase, the addition of a methylene group across the carbon-carbon double bond of mature unsaturated fatty acids results in the formation of cyclopropane fatty acids (Grogan and Cronan, 1997), including methylene hexadecanoic and methylene octadecanoic acids in *E. coli* (Marr and Ingraham, 1962).

In Section 2.5.1 it was noted that the shape of a given phospholipid molecule depends on both the headgroup structure as well as the degree of unsaturation and chain length of the acyl chains (Cronan, 2003). Changes in phospholipid composition and shape, and hence phospholipid-packing properties, are translated into changes in membrane fluidity (or rigidity) and permeability (Xiang and Anderson, 1995). Therefore the primary reason for changes in lipid composition during different bacterial growth phases, and in response to various stresses, is to allow cells to maintain a particular state of fluidity and / or phase behaviour in the membrane bilayer (Russell *et al.*, 1995). Membrane lipids are directly responsible for the passive permeability of the membrane, and are indirectly responsible for other membrane properties via their interactions with membrane proteins (Russell, 1989). Lipids can undergo transitions between disordered (fluid) and ordered (gel) states (Elson *et al.*, 2011), and these transitions can be caused by numerous environmental factors including temperature, pressure, ionic strength, pH, nutrients, chemicals and water activity (Mykytczuk *et al.,* 2007). Active changes in membrane composition work to counteract and / or minimise unfavourable phase transitions (Russell *et al.*, 1995). For example, a less fluid membrane may defend against stress by limiting transport and respiration, both membrane associated phenomenon, thus conserving energy (McGarrity and Armstrong, 1975).

As an oversimplified rule of thumb, phospholipids that contain unsaturated fatty acids have much lower transition temperatures (T_m) than do phospholipids containing saturated fatty acids (Mansilla *et al.*, 2004). This is because saturated fatty acid chains can pack tightly, while the rigid kink of the cis double bond in unsaturated fatty acids results in acyl chain packing that is much less tight (Mansilla *et al.*, 2004). Change in the proportion of unsaturated to saturated fatty acids is the primary mechanism by which bacteria such as *E. coli* regulate membrane fluidity in response to temperature (de Mendoza and Cronan, 1983). At temperatures below the normal physiological range for a particular bacterium, lipid bilayer membranes undergo a reversible change from a fluid (L_α) to a gel (S_0) state (de Mendoza and Cronan, 1983). To counteract this change, proportionally more unsaturated fatty acids are incorporated into the membrane, to maintain membrane fluidity as temperature decreases (de Mendoza and Cronan, 1983).

Beyond the phase transitions of single lipid species, membranes containing a mixture of lipid species can undergo phase separations (Elson *et al.*, 2011). For example, where two phospholipid types are present with different T_m, a homogenous mixture of the two phospholipids above the higher T_m will be transformed to a two-phase mixture of liquid and gel phases between the T_m, and finally to a mixture of two gel components below the lower T_m (Elson et al, 2011). The presence of distinct *E. coli* membrane domains with different order and polarity has been shown using the fluorescent probes Laurdan and 1,3-diphenyl-1,3,5-hexatriene (Vanounou *et al.*, 2002). Such phase separations have long been considered in terms of their ability to affect both local domain (as opposed to bulk membrane) permeability, as well as the function of membrane-associated proteins (enzymes, channels and gates); in their formulation of the fluid mosaic model, Singer and Nicolson (1972) noted heterogeneities at length scales up to 100nm. More recently there has been growing interest in nanodomains, composed of an enriched, ordered liquid phase (L_o) (more tightly packed, viscous and physically distinct from the surrounding L_α lipid phase) and their affect on protein function (Elson *et al.*, 2011).

2.5.4 The cell envelope in the acid context

Membrane integrity has an essential role in protecting cells against low external pH (Jordan *et al.*, 1999). As discussed (*see* Section 2.4.1), a simple model of passive diffusion of undissociated acetic acid molecules into bacterial cells is generally assumed. However, given the hydrophilic, charged character of the outer surface of the outer membrane (which excludes lipophilic molecules), and the hydrophobic lipid regions of both the outer and cytoplasmic membranes (which acts to prevent diffusion of ionic and polar molecules) (*see* Sections 2.5.1 and 2.5.3), it is suggested that any passive diffusion of such acids into bacterial cells must be a complex event. Solubility-diffusion theory, which equates the lipid bilayer membrane with a bulk lipid solvent, might thus be expected to be an oversimplification when describing transport of weak organic acids across cell membranes (Xiang & Anderson, 1997). Further, the permeability of the cell envelope as a whole is rarely considered; it is generally assumed that weak acids are freely permeable across the outer membrane and the periplasm (including the cell wall), and that the

rate limiting step is diffusion across the cytoplasmic membrane. Thus the outer membrane and periplasm are rarely considered in studies of acetic acid sensitivity and resistance.

Although often discussed as a lipophilic species, short chain fatty acids such as acetic acid are in fact polar molecules which are only weakly lipophilic (Steiner and Sauer, 2001). Lu *et al.* (2011) showed that the amount of undissociated acetic acid required to achieve a 5-$\log_{10}$ reduction of *E. coli* O157:H7 was 370 mM higher than that of sorbic acid, despite the almost identical pK_a of the two acids, and attributed this difference in large part to the much greater hydrophobicity and membrane permeability of sorbic acid. Thus it could be expected that changes affecting membrane permeability might be expected to have a greater effect on acetic acid diffusion than on the diffusion of more hydrophobic species, at least across the cytoplasmic membrane.

A major phenomenon that solubility-diffusion theory fails to take into account is the effect of bilayer chain order on the permeability coefficient of weak organic acids (Xiang & Anderson, 1997). The permeability of acetic acid has been shown to be able to be predicted from the free surface area in the lipid bilayer, which is a function of the chain-packing properties, provided that a number of other effects including the bilayer barrier thickness are taken into account (Xiang & Anderson, 1997). As discussed (*see* Section 2.5.3), both the headgroup and the acyl chain composition of membrane phospholipids can affect phospholipid-packing properties. It has been shown that *E. coli* adapted for one doubling time to pH 5.0 have elevated levels of cyclopropane fatty acids, compared with non-acid adapted cells, and that the acid tolerance of individual *E. coli* strains is correlated with their cyclopropane fatty acid content (Brown at al. 1997). Changes in the lipid composition of membranes may affect their proton conductance (Arcisio-Miranda *et al*., 2009), reducing leakage of protons across membranes when the external proton concentration is high (Jordan *et al.*, 1999). Further, changes in lipids can indirectly influence permeability through interaction with membrane proteins (Russell et al., 1995). Changes in membrane permeability have been shown to correlate with changes in protein composition including the K^+/H^+ and Na^+/H^+ antiport systems, the F_1F_0 ATPase, electron transport chains, and numerous solute-proton symport systems (Jordan *et al.,* 1999).

In a rare study investigating the effect of the outer membrane on acetic acid resistance, Barua *et al.* (2002) showed various O-polysaccharide side chain and enterobacterial common antigen (ECA) mutants of *E. coli* O157:H7 and *Salmonella* Typhimurium to be more sensitive under growth-permissive conditions. Barua *et al.* (2002) hypothesised that the fully expressed O-antigen of *E. coli* O157:H7 may lower acetic acid permeability and, further, prevent disruption of the outer membrane during increasing osmotic pressure due to the accumulation of acetate anions in the cell cytoplasm. However Milillo *et al.* (2011) have suggested an alternative interpretation for the observations of Barua *et al.* (2002), in which any damage to the outer membrane (including to LPS) may simply result in increased acid sensitivity. Milillo *et al.* (2011) support this hypothesis with their observations of increased potassium leakage and transmission electron microscopy (TEM) evidence of membrane destabilisation of *Salmonella* Typhimurium on exposure to acidified solutions of weak acid salts.

2.6 Osmolyte effects on the cell envelope

2.6.1 Introduction

Osmolytes are solutes affecting osmosis, i.e. the movement of solvent (water) molecules through a semi-permeable membrane, with the aim of equalising the solute concentration on both sides of the semi-permeable membrane (Haynie, 2001). The net movement of water is from the less concentrated (hypotonic) to the more concentrated (hypertonic) solution (Haynie, 2001). This effect can be countered by increasing the pressure of the hypertonic solution with respect to the hypotonic (Haynie, 2001). The osmotic pressure is defined as the pressure required to maintain an equilibrium, with no net movement of solvent (Haynie, 2001). Osmotic pressure is a colligative property, meaning that the osmotic pressure depends on the molar concentration of the solute but not on its identity (Haynie, 2001).

Bacterial cells are permeable to water, but selectively permeable to extra- and intra-cellular solutes, ideally permitting entry and exit of only those solutes needed for metabolism (Alemohammad & Knowles, 1974). Cells thus act as osmometers (Alemohammad & Knowles,

1974), with changes in external osmotic pressure resulting in the movement of water into or out of the cell, and hence volume changes in the cell so that osmotic equilibrium between the cell and its external environment is maintained (Matts and Knowles, 1971).

Osmolytes may be charged molecules such as salt ions, or uncharged molecules such as sugars (Haynie, 2001). In the case of charged molecules, an extension of the concept of osmotic equilibrium is that of Donnan (or Gibbs-Donnan) equilibrium. The Donnan equilibrium relates to the unequal distribution of electrical charge across a semi-permeable membrane. The electrical potential arising is known as the Donnan potential (Yee *et al.*, 2004). This unequal charge can arise if the solution on one side of the semi-permeable membrane contains an impermeable charged solute, such as a protein anion (Tsujii, 2002; Yee *et al.*, 2004). Thus the solution containing an impermeable anion will always have a higher concentration of permeable cations than the solution that does not contain the impermeable anion (Yee *et al.*, 2004). There is a Donnan equilibrium between the periplasm and the extracellular environment (Tsujii, 2002), and a corollary of this is that an electrical potential exists across the outer membrane; for *E. coli* and *Salmonella* Typhimurium in minimal salts medium this potential was determined as around 30 mV, negative inside (Stock *et al.*, 1977). A significant part of the resistance of bacterial cells against anionic solutes may stem from the fact that the periplasmic space contains many negatively charged macromolecules, and the resulting Donnan equilibrium thus favours exit of permeable anions from the periplasm (Nikaido, 1993).

If the osmolarity of the external environment is increased (i.e. made hypertonic, relative to the cell) by the addition of non-permeable solutes such as NaCl and sucrose, bacteria respond by regulating their intracellular solute concentration via the accumulation of compatible solutes (Beales, 2004). Compatible solutes are osmolytes that are preferentially excluded from the surface of cell macromolecules such as proteins and nucleic acids and are thus compatible with biological function (Arakawa and Timasheff, 1985; Felitsky *et al.*, 2004). The mechanisms of exclusion may include increasing the surface tension of water, steric incompatibility and repulsive forces; irrespective of the mechanism, the result is a general stabilising effect opposing the unfolding / denaturation of macromolecules that might otherwise occur (Sleator and Hill, 2001).

The accumulation of compatible solutes results in an increase in internal osmotic pressure and hence restoration of turgor pressure (Beales, 2004).

Bacterial compatible solutes are synthesised *de novo* or are taken up from the environment, and include K^+, L-proline, glutamate, proline, trehalose and glycine betaine (Beales, 2004). Compatible solutes are highly soluble, pH neutral and are generally the end products of metabolism (Beales, 2004). The cytoplasmic membrane is selectively permeable to compatible solutes, allowing the concentration of the compatible solute pool to be determined by the osmotic pressure (Beales, 2004). A large number of systems are involved in bacterial osmoregulation. For example, in *E. coli* the following osmoregulatory systems are known; aquaporin AqpZ, mediating passive transmembrane water flux, the K^+ transporters TrkA(G/H)/SapD and KdpFABC, mediating K^+ accumulation in response to high osmotic pressure; KdpDE, a two component regulatory system controlling kdpFABC transcription in response to K^+; suppression of glutamate catabolism leading to accumulation of glutamate as a K^+ counterion; trehalase (TreA), hydrolysing extracellular trehalose at high osmotic pressure; OtsA and OtsB, mediating cytoplasmic trehalose synthesis; repression of genes encoding a trehalose catabolic system; transporters ProP, ProU, BetT and BetU, mediating organic osmolyte accumulation at high osmotic pressure; ProQ, regulating ProP; enzymes BetA and BetB, mediating glycine betaine synthesis from choline; and mechanosensitive channels MscL and MscS, mediating solute efflux in response to decreasing osmotic pressure (Romanstov *et al*,. 2009).

2.6.2 Plasmolysis, deplasmolysis and turgor

Plasmolysis is the most dramatic morphological response of bacteria placed into a hypertonic environment (Scheie, 1969). In Section 2.6.1 it was noted that exposure of bacteria to hypertonic solutions results in an immediate loss of water from the cell, which is accompanied by a decrease in cytoplasmic volume (Beales, 2004), and an apparent retraction of the plasma membrane from the cell wall (Scheie, 1969). Plasmolysis resulting from osmotic upshift is generally regarded as a non-lethal and transient phenomenon (Scheie, 1969). Rapid contraction of the cell volume proceeds until an osmotic equilibrium is achieved, and then a longer-term rebalancing of

compatible solutes, often commencing with uptake of K^+, takes place with the ideal of deplasmolysing the cell and restoring cytoplasmic volume (Jovanovich *et al.*, 1988). However under conditions lacking carbon sources and containing only membrane-impermeant solutes (e.g. NaCl and sucrose), active K^+ uptake has been demonstrated to be prevented with the result that cells are effectively locked into a plasmolysed condition (Konopka *et al.*, 2009).

Cell recovery can be dramatically reduced if the plasmolysis is extreme involving for example collapse of the cell wall, and it has also been suggested that survival rates may be related to the interval of time spent in the plasmolysed state (Scheie, 1969). Decad & Nikaido (1976) observed that cytoplasmic volume in Gram negative microorganisms was reduced to ~50% at ~0.3M NaCl but the plasmolysis-induced cell wall damage was minimal, while at 0.5M (2.9% wt/wt) NaCl, cell wall damage was present in a large fraction of cells. Similarly, Scheie (1969) observed slight plasmolysis (usually occurring at only one end of the cell) to be predominant among *E. coli* cells at 0.2M (~7% wt/wt) sucrose, while extensive plasmolysis was predominant at 0.4M (~14% wt/wt), and cell wall collapse evident at concentrations > 0.4M.

It is often assumed that turgor pressure drops primarily across the cytoplasmic membrane, and phase contrast and electron microscopy observations have supported this model (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998). It has been postulated that the initial response of Gram negative bacteria such as *E. coli* and *Salmonella* to osmotic shock is a combined contraction of the entire cell envelope (i.e. outer membrane, cell wall / periplasm and cytoplasmic membrane) to produce a composite wall containing all elements (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998). This wrinkled composite structure may, or may not then split to allow partial straightening and re-extension of the cell wall and outer membrane, thereby enlarging the periplasmic space (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998). Further, studies of the osmoregulation of *kdp*ABC operon expression in *E. coli* have suggested that changes in turgor pressure are sensed by the cytoplasmic membrane-bound kdpD sensor kinase (Laimins *et al.*, 1981).

However, it has also been postulated that the periplasm and cytoplasm are in fact maintained in an iso-osmotic state (Stock *et al.*, 1977). From analysis of periplasmic membrane-derived

oligosaccharides concentrations, and using a fluorescence recovery after photobleaching assay to probe the fluidity of the periplasm as a function of external osmolarity, it has been shown that under hyperosmotic conditions there is a net gain of water by the periplasm as the cytoplasm loses water (Cayley *et al.*, 2000; Sochacki *et al.*, 2011). This observation is consistent with a model in which osmotic pressure is regulated primarily across the cell wall, while the cell actively maintains iso-osmolarity between the periplasm and the cytoplasm (Cayley *et al.*, 2000; Sochacki *et al.*, 2011).

For bacteria hypoosmotic shock generally results in only minor changes in cell volume compared to those induced by hyperosmotic shock (Beales, 2004). Bacterial cell walls are normally subject to outward turgor pressure, enabling them to better withstand a reduction in external osmolarity than are unwalled animal cells (Record *et al.*, 1998). However in the case of large osmotic downshifts, such as following an initial hypertonic challenge, further damage to the cell approximating cytolysis can occur (Vásquez-Laslop *et al.*, 2001). With milder hypoosmotic challenge, *E. coli* responds by reducing cytoplasmic potassium glutamate and trehalose concentrations and replacing K^+ with the divalent cation putrescine to minimise turgor pressure while preserving cytoplasmic electroneutrality (Record *et al.*, 1998).

2.6.3 Effects on the cell-solution interface

At normal physiological pH and ionic strength, bacteria possess a net negative surface charge, primarily resulting from acidic (e.g. carboxyl) groups at the surface of the cell (James, 1991). However, the bacterial cell surface is highly dynamic, responding strongly to changes in its external environment (Poortinga *et al.*, 2002). Charged groups may associate or dissociate with changes in pH and ionic strength (Poortinga *et al.*, 2002). Net negative charges on surfaces tend to hold cations when the surface is placed into solutions of electrolytes (James, 1991).

One generally accepted model for the interaction of cations with bacterial cells that of Stern, embodying the principles of Hemholtz, Perrin, Gouy and Chapman, and describing a diffuse electrical double layer (James, 1991). The first layer is the Stern layer, where ions are held by

specific chemical adsorption or by localised electrostatic interaction in close contact (~ 0.5 nm) with the surface. The electrostatic field at the surface results in a net attraction of ions of the opposite sign (i.e. for a negatively charged cell, a net attraction of cations). The second layer, extending into the liquid phase, is diffuse and, due to ion movement by thermal agitation, is not uniform (James, 1991). On time average, if cations are predominantly associated with the innermost layer, anions will be predominantly associated with the outermost layer (James, 1991). The extent or thickness of the double layer is dependent on the concentration and valency of the ions in the solution. In the case of a negatively charged cell, as the ionic strength of the electrolyte solution is decreased, or the pH increased, the thickness of the ionic atmosphere increases, and the coulombic screening of the charged surface groups is reduced. Thus the cell envelope can be thought of as a series of shells merging into one another, with the outermost shell being an ionic atmosphere held by ionic groups on the cell surface (James, 1991).

It has been suggested that the Stern model has limited physical meaning for bacterial cell surfaces, although it may retain usefulness as an empirical approach to their description (Yee *et al.*, 2004). The Stern model was originally developed for ion-impermeable surfaces, and is unlikely to accurately describe the electric field formed by three-dimensional semi-permeable membranes (Poortinga *et al.*, 2002). An alternate model to that of the Stern model is the Donnan model. For Gram positive bacteria, the "Donnan potential" is defined as the electrical potential within the cell wall, the "surface potential" is the electrical potential at the cell-water interface, and the "zeta potential" is the electrical potential at the shear plane, with the magnitude of the Donnan potential controlled by the fixed charge excess within the cell wall (Yee *et al.*, 2004). For Gram negative bacteria, the Donnan potential is across the outer membrane, between the periplasm and the external environment (Nikaido, 1993).

2.6.4 Effects on the outer membrane

Changes in ionic strength may produce changes in the conformation of the macromolecular species which make up the outer layers of the cell (James, 1991). The ability of the anionic outer membrane to bind cations has been demonstrated with the cationic antimicrobial nisin (Helander

& Mattila-Sandholm, 2000) and the heavy metal ion of cadmium (Hustavova, 1995) both serving to stabilise the physical structure of the outer membrane through electrostatic interactions. Conversely, addition to HEPES buffer of cations (Mg^{2+}, Ca^{2+}, and Na^{+}) before or after *E. coli* and *Salmonella* cell breakage in a French press caused the formation of aggregates of outer membrane material (vesicles) from rough, but not smooth, strains (Stan-Lotter and Sanderson, 1981). The aggregation of outer membrane material by addition of monovalent or divalent cations was found to be reversible (Stan-Lotter and Sanderson, 1981), suggesting disruption of ionic bonds by the introduction of positive charges to be the cause of outer membrane destabilisation (Schindler and Teuber, 1978). It was proposed that the O-antigen of smooth strains of *E. coli* and *Salmonella* might prevent aggregation of the outer membrane by cations by steric hindrance of interactions between cations and negatively charged groups (Stan-Lotter and Sanderson, 1981).

2.6.5 Effects on the periplasm, including the cell wall

As discussed in Section 2.6.2, the lethal effects of hyperosmotic shock have been proposed to be a consequence of gross physical damage to the cell wall (Scheie, 1969; Decad and Nikaido, 1976). In the periplasmic gel model (*see* Section 2.5.2), it is proposed that osmotic shock compresses the gel (Hobot *et al.*, 1984). The lipid bilayer of the outer membrane is closely attached to the cell wall, for example through lipoproteins and via non-covalent interactions with other outer membrane proteins (Duque *et al.*, 2004), However, the poles are ‘free’ of peptidoglycan and present a point of weakness which in turn favours the formation of plasmolysis spaces (Scheie, 1969; Duque *et al.*, 2004; Koch, 1995; Koch, 1998).

2.6.6 Effects on the cytoplasmic membrane

Plasmolysis spaces have been proposed to form in a number of ways including the rearrangement of sections of the cytoplasmic membrane into Scheie (non-cylindrical, rough) or Bayer (tubular) structures to accommodate excess membrane, or by the removal of cytoplasmic membrane material in the form of endocytotic or exocytotic vesicles (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998). Electron micrographs of osmotically shocked cells often reveal wrinkled

cytoplasmic membranes (Beney *et al.*, 2004). It has been proposed that it is the formation of plasmolysis spaces that allows the cell wall and outer membrane to re-extend, following initial contraction on hyperosmotic shock (Koch, 1995; Schwarz and Koch, 1995; Koch, 1998).

Growth at high osmolarities leads to changes in membrane lipid composition, and it has been suggested that this change is part of the osmoregulation sensing mechanism (Russell and Kogut, 1985). For example, McGarrity and Armstrong (1975) examined the effect of NaCl concentration (0, 0.3 and 0.6M) on the phospholipid fatty acid composition of *E. coli* K12, and found significant differences in stationary phase cultures. Growth in media containing high NaCl concentrations resulted in a reduction in the proportion of unsaturated fatty acids and an increase in the proportion of cyclopropane fatty acids (McGarrity and Armstrong, 1975). However, in contrast to the effect on membrane composition of temperature (*see* Section 2.5.3), the major change in response to high concentrations of osmolytes is in the head group of the lipids, with the most common alteration being an increase in the proportion of anionic phospholipids (Russell *et al.*, 1995).

In particular, the proportion of cardiolipin has been shown to increase substantially in response to osmotic stress, irrespective of the osmolyte (Romanstov *et al.,* 2008; Romantsov *et al.*, 2009). As discussed (*see* Section 2.5.3), cardiolipin is formed from the condensation of two phosphatidylglycerol molecules to form cardiolipin and glycerol (Romanstov *et al.*, 2009). However, although cardiolipin is synthesised from phosphatidylglycerol, for *E. coli* the proportion of cardiolipin increases as the proportion of phosphatidylethanolamine decreases, and the proportion of phosphatidylglycerol in fact remains relatively constant under osmotic stress (Romanstov *et al.*, 2009). Both the genes encoding cardiolipin synthase (Cls) and phosphatidylglycerol synthase (*pgsA*) may be osmotically induced (Romanstov *et al.*, 2009).

Finally, exposure of *E. coli* to hyperosmotic shock has been shown to have a deleterious effect on several membrane energy-linked functions (Houssin *et al.,* 1990). Houssin *et al.* (1990) showed that respiration was inhibited at high osmolarity achieved using either NaCl or sucrose (but not glycerol), but that the $\Delta\psi$ remained unaffected. In addition, four different sugar transport systems

have been shown to be inhibited by high osmolarity: the lactose proton symport, the glucose phosphotransferase system, the binding-protein dependent maltose transport system (Roth *et al.,* 1985; Houssin *et al.*, 1990) and the glycerol facilitator (Houssin *et al.*, 1990). While it was hypothesised that inhibition of sugar transport could be a consequence of the abolition of energy sources required for active transport, Houssin *et al.* (1990) confirmed that this was not the case. Roth *et al.* (1995) have suggested that the inhibition is instead caused by conformational changes in the various membrane proteins responsible for these energy-linked functions, arising from more general changes in membrane conformation occurring as a result of plasmolysis.

2.7 Conclusion

Eklund (1989) has stated that, in principle, all important subcellular processes or structures should be investigated for their responses to the action of preservatives such as weak acids. Further, Eklund (1989) has emphasised the importance of using the term ‘modes of action’ as distinct from ‘mode of action’, thus more accurately conveying the expectation that more than one cellular target is likely to be involved, and that inhibition is most likely the result of a combined load of cell perturbations. Even for single preservative hurdles, such as acetic acid, NaCl and sucrose, the modes of action remain partially obscure. In combination, much remains to be learned regarding the interaction of such factors in microbial inactivation. Further, observations of antagonistic effects between preservative factors warrant particular attention, given the strong contemporary use of hurdle technology. As reviewed here, the cell envelope has a central role in the response and resistance to acetic acid and osmotic stress, and is an attractive target for studies into a possible mechanism of osmolyte protection of *E. coli* and *S. enterica* against acetic acid inactivation.

Escherichia coli outer and cytoplasmic
membrane changes during exposure to acetic acid, in response to exposure time, osmolytes, temperature and pH.

The following chapter has been removed for copyright or proprietary reasons

The following have been published as:

Results – Chapter 1 (pp 61-68)

Chapman, B and Jensen, N and Ross, T and Cole, M, Salt, Alone or in Combination with Sucrose, Can Improve the Survival of Escherichia coli O157 (SERL 2) in Model Acidic Sauces, Applied and Environmental Microbiology, 72, (8) pp. 5165-5172. ISSN 0099-2240 (2006)

http://dx.doi.org/10.1128/AEM.02522-05

Abstract

The commercial production of microbiologically safe and stable sauces containing acetic acid is guided by the Comité des Industries des Mayonnaises et Sauces Condimentaires de la Communauté Économique Européenne's (CIMSCEE) code. The CIMSCEE safety value is calculated using a linear regression equation combining weighted contributions of pH and aqueous-phase concentrations of undissociated acetic acid, NaCl, and sugars. By implication, the CIMSCEE safety equation predicts that increasing concentrations of hurdles will always increase inactivation of the target pathogen. In this study, the time to achieve a 3-log10 reduction of an acid-resistant, acid-adapted, Shiga toxin-producing Escherichia coli (STEC) O157 isolate was determined experimentally for 81 formulations at various pHs and acetic acid, NaCl, and sucrose concentrations in a broth model. The combinations were intended to simulate the aqueous phase of acidic sauces and dressings. Experimental data were fitted to the log logistic model to estimate the time to 3-log10 reduction (t3D). Comparison of fitted t3D estimates with CIMSCEE values showed agreement in predicting safety (as defined by CIMSCEE) for the majority of formulations. However, CIMSCEE safety predictions were “fail dangerous” for 13 of 81 formulations. Among these formulations and others, the observed E. coli t3D initially increased and then decreased with increasing osmolalities (NaCl and sucrose). Relative protection increased with exposure time where the protective effect of NaCl predominated. While commercial acidic sauces are not considered high-risk vehicles for STEC, interactions among hurdles that decrease their combined effectiveness are deserving of further investigation because they may reveal mechanisms of broader relevance in the inactivation of pathogens in foods.

Results -Chapter 2 (pp 71-76)

Chapman, B and Ross, T, Escherichia coli and Salmonella enterica are Protected against Acetic Acid, but Not Hydrochloric Acid, by Hypertonicity, Applied and Environmental Microbiology, 75, (11) pp. 3605-3610. ISSN 0099-2240 (2009)

http://dx.doi.org/10.1128/AEM.02462-08

Abstract

Chapman et al. (B. Chapman, N. Jensen, T Ross, and M. B. Cole, Appl. Environ. Microbiol. 72:5165-5172, 2006) demonstrated that an increased NaCl concentration prolongs survival of Escherichia coli O157 SERL 2 in a broth model simulating the aqueous phase of a food dressing or sauce containing acetic acid. We examined the responses of five other E. coli strains and four Salmonella enterica strains to increasing concentrations of NaCl under conditions of lethal acidity and observed that the average "lag" time prior to inactivation decreases in the presence of hydrochloric acid but not in the presence of acetic acid. For E. coli in the presence of acetic acid, the lag time increased with increasing NaCl concentrations up to 2 to 4% at pH 4.0, up to 4 to 6% at pH 3.8, and up to 4 to 7% (wt/wt of water) NaCl at pH 3.6. Salmonella was inactivated more rapidly by combined acetic acid and NaCl stresses than E. coli, but increasing NaCl concentrations still decreased the lag time prior to inactivation in the presence of acetic acid; at pH 4.0 up to 1 to 4% NaCl was protective, and at pH 3.8 up to 1 to 2% NaCl delayed the onset of inactivation. Sublethal injury kinetics suggest that this complex response is a balance between the lethal effects of acetic acid, against which NaCl is apparently protective, and the lethal effects of the NaCl itself. Compared against 3% NaCl, 10% (wt/wt of water) sucrose with 0.5% NaCl (which has similar osmotic potential) was found to be equally protective against adverse acetic acid conditions. We propose that hypertonicity may directly affect the rate of diffusion of acetic acid into cells and hence cell survival.

Results – Chapter 3 (pp 79-90)

Chapman, B and Scurrah, KJ and Ross, T, Contemporary formulation and distribution practices for cold-filled acid products: Australian industry survey and modelling of published pathogen inactivation data, Journal of Food Protection, 73, (5) pp. 895-906. ISSN 0362-028X (2010)

Abstract

A survey of 12 Australian manufacturers indicated that mild-tasting acids and preservatives are used to partially replace acetic acid in cold-filled acid dressings and sauces. In contrast to traditional ambient temperature distribution practices, some manufacturers indicated that they supply the food service sector with cold-filled acid products prechilled for incorporation into ready-to-eat foods. The Comité des Industries des Mayonnaises et Sauces Condimentaires de la Communauté Économique Européenne (CIMSCEE) Code, a formulation guideline used by the industry to predict the safety of cold-filled acid formulations with respect to Salmonella enterica and Escherichia coli, does not extend to the use of acids and preservatives other than acetic acid nor does it consider the effects of chill distribution. We found insufficient data in the published literature to comprehensively model the response of S. enterica and E. coli to all of the predictor variables (i.e., pH, acetic acid, NaCl, sugars, other acids, preservatives, and storage temperature) of relevance for contemporary cold-filled acid products in Australia. In particular, we noted a lack of inactivation data for S. enterica at aqueous-phase NaCl concentrations of >3% (wt/wt). However, our simple models clearly identified pH and 1/absolute temperature of storage as the most important variables generally determining inactivation. To develop robust models to predict the effect of contemporary formulation and storage variables on product safety, additional empirical data are required. Until such models are available, our results support challenge testing of cold-filled acid products to ascertain their safety, as suggested by the CIMSCEE, but suggest consideration of challenging with both E. coli and S. enterica at incubation temperatures relevant to intended product distribution temperatures.

6 Manuscript 4: Membrane changes with acetic, time, osmolytes, cold and pH

Chapman, B., L. Turnbull, C.B. Whitchurch, and T. Ross. *Escherichia coli* outer and cytoplasmic membrane changes during exposure to acetic acid, in response to exposure time, osmolytes, temperature and pH.

In preparation

Escherichia coli outer and cytoplasmic membrane changes during exposure to acetic acid, in response to exposure time, osmolytes, temperature and pH

Running title – Membranes with acetic, time, osmolytes, cold & pH

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KEYWORDS

E. coli; acetic acid; salt; sucrose; cold; pH; membrane; flow cytometry, 3D-SIM

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The inactivation response of *Escherichia coli* to increasing osmolyte concentration is non-monotonic in the presence of acetic acid, and was hypothesised (B. Chapman and T. Ross, Appl. Environ. Microbiol. 75:3605-3610, 2009) to be partly due to differences in changes to membranes during such exposure. Here we report changes in *E. coli* outer and cytoplasmic membranes during exposure to acetic acid. Outer membrane permeability, determined using sensitisation to crystal violet and hexidium iodide (HI) permeability, increased with exposure time. Cytoplasmic membrane damage was not observed using "live / dead" staining with SYTO® 9 and propidium iodide. However, it was noted that SYTO® 9-staining also increased with exposure time. Crystal violet sensitivity and SYTO® 9 fluorescence increases were non-monotonic with respect to osmolyte concentration, largely independent of osmolyte type. Changes correlated with decreased pH and were lessened by colder temperature. Three Dimensional Structured Illumination Microscopy (3D-SIM) suggested increases in SYTO® 9- and HI-staining may be partly due to high affinity staining of specific cytoplasmic membrane regions. These domains are initially localised in polar regions but more numerous and widely distributed with increasing exposure time. The presence of the anionic phospholipid cardiolipin in these domains was confirmed using nonyl acridine orange (NAO) staining. Cell plasmolysis was also observed. Collectively, the results suggest possible membrane changes that could contribute to the non-monotonic *E. coli* inactivation response.

The inactivation response of *Escherichia coli* to acetic acid is non-monotonic under conditions of increasing osmolarity imposed by either NaCl alone or NaCl and sucrose (8, 9). Specifically, increasing osmolarity initially delays inactivation but increases subsequent inactivation rates so that survival is optimal at intermediate hypertonic osmolarities. *E. coli* survival in the presence of acetic acid and osmolytes has also been shown to be affected by pH / acetic acid concentration (8, 9, 10) and by storage temperature (10).

The antibacterial activity of acetic acid is traditionally explained by weak acid theory, which states that acetic acid in its undissociated, lipophilic form will cross the cytoplasmic membrane and then dissociate according to pKa to acidify the cytoplasm (18). Neutrophiles such as *E. coli* actively work to counter cytoplasmic acidification via a number of passive and active mechanisms collectively termed pH homeostasis (6). An intact cytoplasmic membrane is regarded as an essential, if generally passive contributor to pH homeostasis (29). For injured bacteria that may recover, an intact cytoplasmic membrane is essential to maintain internal metabolite pools until favourable conditions are encountered to resume growth (22).

In an earlier study (9) we observed a non-monotonic pattern of outer membrane damage among the recoverable *E. coli* cell population in response to increasing NaCl concentration in the presence of acetic acid, paralleling the inactivation response. Outer membrane damage increased with exposure time at all NaCl

concentrations but appeared earliest in the presence of "high" (initially hypertonic) NaCl concentrations, and earlier in the presence of "low" (hypotonic and isotonic) than "intermediate" (hypertonic) NaCl concentrations. The cause of outer membrane damage in these test systems was not explicitly determined but it was noted that, in contrast to the non-monotonic pattern observed in the presence of acetic acid, membrane damage in response to increasing NaCl concentrations only increased monotonically in the presence of hydrochloric acid over the time course of the experiments.

Outer membrane damage might arise under initially hypertonic test conditions as a consequence of changes to the physical structure of the cytoplasmic membrane. When placed in a hypertonic medium, rapid loss of water from the cytoplasm can result in a withdrawal of the cytoplasmic membrane from the cell wall in a physicochemical response known as plasmolysis (33). The exact nature (if any) of the mechanical relationship between the cytoplasmic membrane and the cell wall of Gram negative bacteria remains unresolved (35), but the cell wall is known to be connected to the outer membrane by Braun's lipoproteins (7). Indirect damage to the outer membrane may occur if the cytoplasmic membrane pulls on anchor points on the thin cell wall of Gram-negative bacteria (4), or on protein complexes that span the periplasmic space (27).

Plasmolysis resulting from osmotic upshift is generally regarded to be a transient phenomenon (33). Rapid contraction of the cell volume proceeds until an osmotic

equilibrium is achieved, and then a longer-term rebalancing of compatible solutes such as potassium, glutamate, proline, trehalose and glycine betaine takes place with the ideal of restoring cell volume (19). However under conditions lacking carbon sources and containing only membrane-impermeant solutes (NaCl and sucrose), active K^+ uptake has been demonstrated to be prohibited with the result that cells are effectively locked into a plasmolysed condition (21). Cell recovery can be dramatically reduced if the plasmolysis is extreme involving, for example, collapse of the cell wall (33). It has also been suggested that survival rates may be related to the interval of time spent in the plasmolysed state (33). It is unknown whether the interval of time spent in the plasmolysed state increases cytoplasmic or outer membrane damage.

Outer membrane damage might also arise in advance of damage to the cytoplasmic membrane, and potentiate the latter. Few studies have examined the ability of acetic acid to damage the outer membrane of Gram-negative bacteria. While lactic and hydrochloric acid have been shown to disrupt the outer membrane (1), it has been reported that acetic acid does not disrupt the outer membrane sufficiently to potentiate the effect of nisin (14). However, damage to the outer membrane of Gram-negative bacteria by e.g., EDTA, is known to increase the osmotic fragility of the cytoplasmic membrane, a fact exploited in the extraction of both periplasmic (28) and cytoplasmic enzymes from *E. coli* spheroplasts by osmotic downshift (37). For the conditions previously tested (8, 9) it is recognised that if acetic acid does cause outer membrane damage, cells

exposed to "high" NaCl concentrations might be additionally injured during recovery by plating, since these cells are exposed to osmotic downshifts on dilution. However the increase in outer membrane damage observed at "low" (including hypotonic) NaCl conditions takes place over many hours, and thus appears inconsistent with a pattern of cytoplasmic damage caused by osmotic downshift of the inoculum, which occurs within seconds of exposure to the hypotonic environment (37).

Coupled with an increase in cytoplasmic membrane damage at "high" osmolarities, a decrease in acetic acid permeability at milder hypertonic osmolarities could provide at least a partial explanation for the observed non-monotonic nature of the *E. coli* inactivation response (8, 9). Under isotonic conditions, the phospholipid bilayer of *E. coli* is in a fluid, lamellar, liquid-crystalline phase, but the phospholipids have been shown to undergo phase transition to a gel state with increasing osmolarity (26). Weak acid theory does not generally take account of cytoplasmic membrane changes on acetic acid permeability, despite observation in other membrane systems of the sensitivity of acetic acid permeability to membrane chain ordering (38). However, it has long been proposed that changes in the physical state of the cytoplasmic membrane might modify the rate at which a given substance could permeate the membrane (15). Changes in the structure (e.g., by sustained plasmolysis) or composition of the cytoplasmic membrane could affect undissociated acid, proton and/or anion permeability. Such changes might then have implications for the rate of

cytoplasm acidification, as well as for the additional proposed mechanisms of acetic acid inactivation, namely uncoupling (3) and acetate anion accumulation (32).

To begin to assess the above hypothesis, the effect of osmolyte concentration and type (NaCl, sucrose) on *E. coli* membrane integrity during prolonged exposure to acetic acid was determined. Membrane changes in response to a limited range of storage temperatures and pH were also assessed.

To assess previous observations of the non-monotonic nature of outer membrane damage, a sensitisation to crystal violet assay of culturable cells was employed, and considered both sucrose and NaCl as osmolytes. To obviate the potential for additional membrane damage during enumeration procedures involving dilution and plating techniques, assessment of outer membrane damage was studied using flow cytometry with the fluorescent indicator hexidium iodide (HI) which is generally regarded as outer membrane impermeant, and therefore not expected to stain *E. coli* (13, 24). Thus uptake of HI by Gram-negative cells infers that outer membrane damage has occurred (13, 24). Cytoplasmic membrane integrity was also assessed by flow cytometry using the fluorescent indicators propidium iodide (PI) and SYTO® 9.

To aid in interpretation of the results of SYTO® 9- and HI- staining, we used super resolution Three Dimensional Structured Illumination Microscopy (3D-SIM) to

visualize the location of SYTO® 9 and HI fluorescence in cells. This revealed brightly staining membrane domains which we hypothesised to be enriched in cardiolipin. This hypothesis was assessed by staining with the cardiolipin-specific fluorescent indicator 10-*N*-nonyl acridine orange (NAO) (25).

MATERIALS AND METHODS

Bacterial cultures and inoculum preparation. Non-pathogenic *E. coli* strain FRRB (Food Research Ryde Bacterial culture collection) 2699 was maintained as a glycerol stock at -80°C, and revived by transferring into 10 ml of NB incubated for 22 h (+/- 1 h) at 37°C (+/-1°C). Aliquots, 10 µl, of the 22 h NB cultures were transferred to 10 ml tryptone soy broth (TSB; Oxoid, CM0129) containing 1% total glucose (TSB1%G), and incubated with shaking at 200 rpm for 22 h (+/- 1 h) at 37°C (+/-1°C). At the conclusion of incubation, the pH of the TSB1%G cultures was determined using pH indicator papers (Type CS, pH 3.8 – 5.5; Whatman International Ltd, UK) to ensure that it was approximately 4.2 (+/- 0.1), indicating that acid conditioning of the cells had occurred.

Experimental treatments. Membrane changes in response to osmolarity and osmolyte type were assessed in twelve acidified Nutrient Broth (NB; CM001; Oxoid, UK) formulations (Table 1) sampled after exposure times of 2, 24, 48, 72 and 120 h (+/- 1 h). Eight formulations (J, A, B, C, D, E, F, G) contained NaCl (Sigma Chemical Co., USA) only, and four formulations (AS, BS, H, I) contained

sucrose (food grade; CSR Sugar, Australia) and NaCl (Table 1). Calculated osmolarities for the twelve treatments were between 0.17 and 2.33 OsM (Table 1); contributions to osmolarity by acids and components of the NB base apart from NaCl (0.5% w/v) were ignored in osmolarity calculations. Two pairs of treatments, A and AS, and B and BS, were formulated to achieve the same calculated osmolarity, arising from either NaCl (A and B) or predominantly from sucrose (AS and BS) (Table 1). Formulations were acidified with glacial acetic acid (Sigma Chemical Co., USA), and the pH adjusted to 3.8 using a Beckman model 390 pH meter with probe 511080 (Beckman Coulter, Inc., USA). NB formulations were filter-sterilized (0.22 μm; Millipore, USA), and aseptically dispensed into sterile 28 ml screw-capped polypropylene containers and equilibrated overnight to 23°C (+/-1°C) prior to inoculation for experiments.

The effects of pH (3.6 and 4.0) and cold temperature (5°C, at pH 3.8) on *E. coli* membrane damage were also assessed for a selection of treatments. Specifically, the effect of pH was assessed using Formulations J, A, B, BS, C and E (Table 1) after exposure times of 2, 24, 48 and 72 h (+/- 1 h). The effect of cold temperature was assessed using Formulations J, A, AS, C, E and G (Table 1) after exposure times of 24, 48, 72 and 120 h (+/- 1 h). In addition to acidified treatments, the effect of high osmolarity (Formulations E, F and G) alone on *E. coli* outer membrane damage and cytoplasmic membrane integrity was determined in non-acidified NB after an exposure time of 2 h only.

Treatment inoculation and incubation. For all experiments except those involving 3D-SIM (*see* below), duplicate individually grown TSB1%G cultures were used to inoculate 20 ml lots of NB formulations to an initial cell concentration of ~10^7 CFU/g. For experiments involving 3D-SIM, TSB1%G cultures were concentrated 10-fold by centrifugation (4800 *g* for 10 min at 23°C) and resuspension of the pellet in 1/10^{th} volume of supernatant prior to inoculation, to achieve sufficient cell density for microscopic observation.

Inoculated treatment broths were incubated statically at 23°C (+/-1°C), except for cold storage experiments, for which inoculated samples were gradually cooled from 23°C to 5°C (+/-1°C) over a period of 2 h in a programmable incubator (APT.line® KB incubator; Binder Inc., USA), and then maintained at 5°C (+/-1°C) for the remainder of the experiment. Samples from 5°C treatments were allowed to equilibrate for 30 min in a 23°C water bath prior to staining for fluorescence based assessments, in order that fluorescence intensity would not be compromised by staining at low temperature.

Crystal violet assay of outer membrane damage. Damage to the outer membrane of culturable cells was assessed by sensitisation to crystal violet. *E. coli* were prepared and inoculated into NB formulations as described above, as well as into a parallel set of broths supplemented with filter-sterilised crystal violet solution (2% w/v; Oxoid, UK), to a final concentration of 0.02%. For each treatment at each sampling time, a 1 ml sample of NB formulation +/- crystal

violet was withdrawn, and decimally diluted twice in buffered peptone water (BPW, Oxoid, CM0509). Dilutions were surface plated (0.1 ml) onto duplicate tryptone soy agar (TSA; Oxoid, CM0131) plates. Plates were incubated aerobically at 37°C (± 1°) for 48 h prior to counting. The proportion of culturable cells with outer membrane damage was inferred by comparing the proportion counts of cells from formulations supplemented with crystal violet to those obtained from NB formulations without crystal violet.

Flow cytometry. Flow cytometric analysis of HI, PI and SYTO® 9-staining was performed using a Becton Dickinson FACSCalibur™ flow cytometer (BD Biosciences, New Jersey, USA), equipped with a 15mW 488 nm air-cooled argon laser for excitation of the fluorescent dyes. Fluorescence was collected using green (FL1, 530/30 nm band pass), orange (FL2, 585/42 band pass) and red (FL3, 670 nm long pass) filters respectively for SYTO® 9-, HI- and PI-stained populations. Osmosol (Lab Aids Pty Ltd, Sydney, Australia) (180 meq/L sodium, 153 meq/L chloride, 5.1 meq/L potassium, 1.0 meq/L EDTA) was used as the sheath fluid for all experiments. For all experiments, events were collected at a low flow rate corresponding to ≤ 1000 events / s, and 50,000 events were collected in each analysis. Forward scatter (FSC), side scatter (SSC) and fluorescence measurements were recorded for each event, logarithmically amplified, and converted into digital signals for further analysis using FlowJo software (Tree Star, Inc., Oregon, USA) software.

Event populations representing bacteria were discriminated and gated in bivariate intensity plots of FSC versus SSC. Although the use of Osmosol resulted in a refractive index mismatch between the sheath fluid and the sample for some formulations, causing an increase in the FSC signal with increasing osmolarity, bacterial cells were easily discriminated from background noise on the basis of FSC and SSC in all cases. Fluorescence analysis was performed on gated populations using histograms and bivariate intensity plots, as appropriate.

HI assay of outer membrane damage. Outer membrane damage was assessed using HI (Invitrogen, USA) and flow cytometry as described above. At each sampling time, 0.5 ml aliquots of inoculated treatments were aseptically withdrawn and mixed with 20 µL of HI solution (2.5 mM in water) to give a final concentration of 100 µM HI. Samples containing HI were incubated in the dark at room temperature (approx. 23°C) for 15 min to allow staining to occur, prior to flow cytometric analysis.

For each treatment at each sampling time, the proportion of cells with outer membrane damage was inferred from those cells (that appeared as a distinct subpopulation) with FL2 fluorescence intensity > 38 units (confirmed by HI staining of *E. coli* with intact outer membranes, under conditions of neutral pH and isotonicity (data not shown)).

PI and SYTO® 9 assay of cytoplasmic +/- outer membrane damage. Cytoplasmic membrane damage was assessed using the commercially available LIVE/DEAD *Bac*Light™ kit (Invitrogen, USA) containing PI and SYTO® 9, and flow cytometry as described above. At each sampling time, 0.5 ml aliquots of inoculated treatments were aseptically withdrawn and mixed with 5 µL of PI solution (240 µM in water) to give a final concentration of 12 µM PI, and 5 µl of SYTO® 9 solution (120 µM in water) to give a final concentration of 1.2 µM SYTO® 9 (i.e. 10:1 PI: SYTO® 9 staining ratio). Stained samples were incubated in the dark at room temperature (approx. 23°C) for 15 min to allow staining to occur, prior to flow cytometric analysis.

For each treatment at each sampling time, the proportion of cells with compromised cytoplasmic membrane integrity was inferred from those cells (that appeared as a distinct subpopulation) with FL3 fluorescence intensity >100 units (confirmed by examination of PI-stained heat-killed *E. coli* (data not shown)).

Following observation of temporal changes in SYTO® 9-fluorescence intensity during dual staining with PI, single stain assays were also undertaken individually with PI and SYTO® 9. Changes in SYTO® 9- and PI-fluorescence in single stain assays was determined from the proportion of cells counted in each of two distinct subpopulations that were apparent, i.e., with fluorescence intensity < or >100 units.

Statistical analysis. Statistical comparisons of the effects of exposure time and osmolytes on membrane damage were made by two-factor analysis of variance ($p \leq 0.05$) using the ANOVA: Two-factor with replication tool of Microsoft® Excel. Specifically, comparisons were made on the basis of the concentrations of NaCl and sucrose (independently), osmolarity, and solute type (comparing formulations A with AS, and B with BS). Details of two-factor ANOVA analyses relating to the effects of time and osmolytes are summarised in the Supplemental material (Table S1).

Statistical comparisons of the effects on membrane damage of exposure time and storage temperature and exposure time and pH were made by two-factor analysis of variance ($p \leq 0.05$) using the ANOVA: Two-factor with replication tool of Microsoft® Excel, as summarised in the Supplemental material (Table S2). Where valid, further statistical comparisons involving pH were made using two factor ANOVA between NB formulations at pH 3.6 and pH 3.8, and between NB formulations at pH 3.8 and pH 4.0.

The effects of NaCl concentration and osmolarity at each exposure time were also assessed by single factor analysis of variance ($p \leq 0.05$) using the single factor ANOVA tool of Microsoft® Excel. Where valid, further statistical comparisons were made using single factor ANOVA between NB formulations at a given sample time for some membrane assays.

3D-SIM visualisation of staining by HI, SYTO® 9 and NAO. *E. coli* cells from a selection of treatments (J, A, C, E and G after 4, 24, 48 and 72 h) were examined by 3D-SIM (12, 34), using a DeltaVisionOMX™ 3D-SIM imaging system (Applied Precision, Issaquah, Washington, USA) following staining with SYTO® 9 and HI and NAO. Stains were applied separately.

For SYTO® 9- and HI -staining, 50 µL aliquots of inoculated treatments were aseptically withdrawn and mixed with 5 µL of SYTO® 9 or HI solution prepared as described above. Aliquots (5 µL) of stained cell suspensions were transferred to poly-L-lysine (Sigma, USA) coated coverslips and gently dried at 30°C on a warming pad for 15 minutes. Cells were mounted in 5 µL Vectashield® (Vector Laboratories, Inc., California, USA) and coverslips placed onto slides and sealed.

For NAO staining, 0.5 ml aliquots of inoculated treatments were aseptically withdrawn and mixed with 0.5 µL of NAO (Invitrogen, USA) solution (200 µM in dimethyl sulfoxide (DMSO; Sigma, USA) to give a final concentration of 200 nM NAO), and incubated at room temperature (approx. 23°C) for 1 h (16). NAO-stained cells did not settle on poly-L-lysine or uncoated coverslips. Therefore, NAO-stained cells were pelleted at the end of staining by centrifugation (4800 *g* for 10 min at 23°C). Pelleted cells were resuspended in the same volume of the appropriate, fresh, NB formulation to which 0.5% w/v intermediate melting temperature agarose (Metaphor, USA) had been added. Agarose was melted by brief microwaving, and NB formulations were cooled to ~ 40°C prior to cell

resuspension. Aliquots, 5 µL, of stained cell suspensions were then transferred to coverslips, and mixed with 5 µL aliquots of Vectashield®. Slides were added, and samples allowed to set prior to examination by 3D-SIM.

Slides were examined by 3D-SIM within 1 – 2 h of preparation. All dyes were excited at 488 nm. Raw images for SYTO® 9- and HI -staining were collected with emission filter sets of 500-550 nm and 608-648 nm, respectively. Raw images for NAO staining were collected at both 500-550 nm and 608-648 nm emission; the interaction of NAO with cardiolipin can result in dual fluorescence (25). Unfortunately the relatively long exposure times required results in bleaching of fluorophores, and therefore it was not possible to capture both 500-550 nm and 608-648 nm emission of NAO for a single image. Images were reconstructed using SoftWoRx v4.5.0 software (Applied Precision Inc., Issaquah, WA) and prepared for presentation using IMARIS v 7.2 software (Bitplane Inc, Zurich).

RESULTS

Crystal violet assay of outer membrane damage. The effects of time and osmolytes on outer membrane damage of culturable cells are shown in Tables 2 and 3 and in Figure 1. Time and NaCl concentration, sucrose concentration, and osmolarity had significant ($p \leq 0.05$) effects on *E. coli* outer membrane damage as assayed by crystal violet sensitisation (Table 2). Outer membrane damage as assayed by crystal violet was significantly dependent on osmolyte type at 0.34

OsM (i.e. paired formulations A and AS), but was not at 0.68 OsM (paired formulations B and BS) (Table 2).

Single factor ANOVA showed a statistically significant ($p \leq 0.05$) effect of both NaCl concentration and osmolarity at all sample times from 2 to 120 h (Table 3). In general, outer membrane damage increased with increasing exposure time (Figure 1). Exposure to acetic acid for 2h at pH 3.8 at high osmolarity (≥ 1.99 OsM) resulted in a statistically significant increase in *E. coli* outer membrane damage compared with exposure at lower osmolarities (Fig. 1a). At 24 h exposure (Fig. 1b), a statistically significant non-monotonic response to increasing osmolarity became apparent, and this trend persisted at 72 h (Fig. 1c).

There was no statistically significant ($p \leq 0.05$) effect of time on crystal violet sensitivity in samples stored at 5°C compared with those stored at 23°C (Table 4), except for formulation AS containing sucrose. However, temperature had a statistically significant effect on crystal violet sensitivity for most of the NB formulations tested, with a smaller average proportion of the culturable cell population showing outer membrane damage at 5°C (Table 4).

pH had a significant ($p \leq 0.05$) effect on outer membrane damage to culturable cells, except for formulation C, with a smaller proportion on average of the population showing damage with increasing pH (Table 5). Outer membrane damage to *E. coli* cells occurred in the presence of high NaCl concentrations at

neutral pH (Figure 2). However, after 2 h exposure to high osmolarity ≥ 1.99 OsM at neutral pH, significantly ($p \leq 0.05$) fewer *E. coli* cells were sensitised to crystal violet (Fig. 2a) in comparison those exposed to pH 3.8 (Fig. 1a).

HI assay of outer membrane damage. Example fluorescence intensity histograms for HI-only staining of *E. coli* cells are shown in Figure 3. As illustrated, large increases in the modal value of fluorescence intensity (FL2) with increasing exposure time was observed for all treatments stored at 23°C (Fig. 3a – 3e). In some cases (see, Fig. 3b, Fig. 3c), the HI (FL2) fluorescence histograms appear bimodal. In a small number of cases, apparently trimodal distributions were observed (data not shown). Thus the overall increase in HI fluorescence intensity with increasing exposure time can be interpreted to arise from a transition of a population of cells with relatively low outer membrane permeability (e.g., Fig. 3a) to a population with relatively high, and more narrowly distributed outer membrane permeability to HI (e.g., Fig. 3e).

The effects of time and osmolytes on outer membrane damage as assessed by HI are shown in Tables 2 and 3 and in Figure 4. The statistical significance of time and NaCl concentration, sucrose concentration, and osmolarity on *E. coli* outer membrane damage as assayed by HI essentially mirrors that assessed by crystal violet sensitisation (Table 2). In contrast to crystal violet, outer membrane damage as assayed by HI depended significantly on osmolyte type at 0.68 OsM

(i.e. paired formulations B and BS), but not at 0.34 OsM (paired formulations A and AS) (Table 2).

HI-fluorescence substantially increased with increasing exposure time (Fig. 4a). However, in comparison with the effect of exposure time, the effect of osmolarity on HI fluorescence is less clear. Single factor ANOVA showed a statistically significant ($p \leq 0.05$) effect of NaCl concentration and osmolarity only at 24 h exposure (Table 3), with increased outer membrane damage apparent for cells exposed to higher osmolarities (Fig. 4a). After 48 h exposure, the average proportion of *E. coli* cells with HI-fluorescence > 38 units appeared non-monotonic (i.e. initially decreasing, than increasing) in response to increasing osmolarity (Fig. 4a), but the trend was not significant (i.e., $p > 0.05$) (Table 2).

The effects of storage temperature and pH on the extent or rate of change of HI staining of *E. coli* cells are exemplified in Fig. 4b. Storage temperature had a significant effect on HI-staining of *E. coli* cells for all of the NB formulations tested, with cells incubated at 23°C taking up significantly more stain, inferring greater outer membrane permeability, than those stored at 5°C (Table 4). Significantly less HI staining was observed at pH 3.8 compared with pH 3.6 for all NB formulations, but HI-staining of *E. coli* at pH 4.0 was not significantly different from staining at pH 3.8 (Table 5). High variability was observed for HI-staining of *E. coli* cells at pH 4.0 (e.g., Fig. 4b). As assessed by HI fluorescence, very little damage occurred to *E. coli* outer membranes in the presence of high NaCl

concentrations at neutral pH, with only around 5% of *E. coli* cells showing fluorescence > 38 units (Fig. 2b).

PI and SYTO® 9 assay of cytoplasmic +/- outer membrane damage. Very little cytoplasmic membrane damage (i.e. little PI-staining) was evident under any of the treatment conditions, regardless of whether the dual staining (with SYTO® 9) or single staining procedure was employed. Even after 120 h exposure to high osmolarities ≥ 1.68 OsM at pH 3.8 and 23°C, less than 8% of *E. coli* cells, on average, were discriminated by PI staining (Figure 5). In comparison with the dual stain assay, assessment of cytoplasmic membrane damage using PI alone resulted in even lower estimates of the percentage of damaged *E. coli* (data not shown).

Average cytoplasmic membrane damage as assessed by PI-staining was significantly less for half of the NB formulations stored at 5°C compared with those stored at 23°C (Table 4). For most NB formulations there was a significant decrease in the average proportion of PI-stained cells at both pH 3.6 and pH 4.0 (Table 5).

Representative fluorescence intensity histograms for SYTO® 9-only staining of *E. coli* populations are presented in Figure 3. As exemplified in Figure 3f - j, and consistent with the results described above for HI-staining, large increases in the mode of the FL1 fluorescence intensity (e.g., compare Fig. 3f and Fig. 3j), and

bimodal distributions (e.g., Fig. 3h) were observed with increasing exposure time for all treatments incubated at 23°C.

The effects of time and osmolytes on SYTO® 9-fluorescence are shown in Tables 2 and 3 and in Figure 6. Time and NaCl concentration, sucrose concentration, and osmolarity had significant ($p \leq 0.05$) effects on SYTO® 9-fluorescence (Table 2). As for HI-fluorescence, SYTO® 9-fluorescence was significantly dependent on osmolyte type at 0.68 OsM (i.e. paired formulations B and BS), but was not at 0.34 OsM (paired formulations A and AS) (Table 2).

Single factor ANOVA showed a statistically significant ($p \leq 0.05$) effect of both NaCl concentration and osmolarity on SYTO® 9-fluorescence at sample times from 24 to 120 h, but not at 2h (Table 3). As observed for HI, SYTO® 9 fluorescence increased with increasing exposure time (Fig. 6a). After 24 h exposure, a non-monotonic response to increasing osmolarity was apparent (Fig. 6a).

The effects of storage temperature and pH on the extent or rate of change of SYTO® 9-staining of *E. coli* cells are exemplified in Fig. 6b. As observed for HI, storage temperature had a significant effect on SYTO® 9-staining of *E. coli* cells for all of the NB formulations tested, with cells incubated at 23°C exhibiting significantly more fluorescence than those stored at 5°C (Table 4 and Fig. 6b). In contrast with HI-staining trends, significantly less SYTO® 9-staining was observed

at pH 4.0 compared with pH 3.8 for all NB formulations tested (Table 5), although a large amount of variability among pH 4.0 samples was observed (Fig. 6b).

3D-SIM visualisation of staining by SYTO® 9, HI and NAO. Representative 3D-SIM images of SYTO® 9-stained *E. coli* cells exposed to different osmolarities for different lengths of time are shown in Figure 7. The most striking observation was the appearance in most samples of brighter green fluorescent regions, apparently located in the plane of the cytoplasmic membrane, contrasting with a less bright diffuse staining of the whole cell. The brighter green staining regions were particularly obvious at the cell poles (usually one pole). These brighter SYTO® 9-staining regions appeared earliest at high osmolarity (compare Fig. 7a and 7e), but were also observed under hypotonic conditions (Fig. 7a and 7b). With increasing treatment duration the contrast between the very bright and more diffusely stained regions was decreased (e.g. compare Fig. 7e and Fig. 7f). Increased staining intensity appeared to arise from a gradual proliferation and distribution of the bright staining regions away from the poles, throughout the cylindrical region of the cell. The decrease in contrast appeared to lag for cells exposed to intermediate osmolarities (compare Fig. 7b and 7f at 24h, with Fig 7d at 72h).

Close detail of the pattern of SYTO® 9- and HI-staining of *E. coli* cells is shown in Figure 8. The appearance of brighter cytoplasmic membrane domains is emphasised in Figs. 8a – 8d, for both fluorescent dyes. Variation in the number

and position of brighter-stained cytoplasmic membrane domains is visible, with some cells presenting as brightly stained over the majority of their surface. For example, most cells in Fig. 8a and 8c show bright patches of staining, while most cells in Fig. 8e are more homogenously stained. In most samples, unstained plasmolysis spaces (18) were also observed in at least some cells, providing cells with an irregular, damaged and occasionally twisted appearance (e.g. Fig. 8e and 8f).

The staining patterns observed with NAO (Figure 9) strongly paralleled those observed for SYTO® 9 and HI. NAO interaction with the cytoplasmic membrane resulted in both green (Fig. 9a – 9d) and red (Fig. 9e and 9f) fluorescence emission. Extremes of the distribution of bright staining regions is emphasised in Fig. 9b, where one cell (to the right of the image) shows two small discrete regions of bright staining while a second cell (to the left of the image) shows overall bright staining. Unstained areas were clearly apparent in polar (Fig. 9f), side-wall (Fig. 9a, 9b and 9d) and septal (Fig. 9c) cell regions, and these are assumed to represent areas of plasmolysis, where the fluorescently-stained cytoplasmic membrane has separated from the remainder of the cell envelope. While more frequently observed in formulations calculated (Table 1) to be initially hypertonic, plasmolysis spaces were also observed among cells exposed to initially isotonic (Fig. 9b, 9f) conditions.

DISCUSSION

A variety of methods were used to assess outer and cytoplasmic membrane damage to *E. coli* exposed to acetic acid in the pH range 3.6 – 4.0, at osmolarities of 0.17 – 2.33 OsM due to sucrose and/or NaCl, and at 5 or 23°C.

As previously assessed by sensitisation to bile salts (9), significant temporal increases in *E. coli* sensitisation to crystal violet and HI-staining strongly support the conclusion that prolonged exposure to acetic acid alters outer membrane permeability of both the culturable and non-culturable (presumed inactivated) population. Similar to previously described inactivation responses of *E. coli* in the presence of acetic acid (9), the sensitivity to crystal violet of the recoverable fraction of *E. coli* was significantly non-monotonic with increasing osmolarity at several exposure times, and was largely independent of solute type. While a similar non-monotonic trend was observed after 48h exposure for HI, this was not statistically significant, and appeared more dependent on solute type. Neverthless, two factor analysis of variance showed a significant effect of osmolarity and individual solute concentrations on HI-staining in the presence of acetic acid.

While both crystal violet sensitisation and HI-staining detect changes in outer membrane permeability, the results collectively suggest that the mechanism of observed changes may be different, or else confounded by other cell changes.

3D-SIM examination suggests that interpretation of HI-staining may be complicated by accumulation in specific cytoplasmic membrane domains. This is discussed further below, together with discussion of SYTO® 9-staining.

PI can only enter and bind to DNA and RNA of cells with physically compromised cytoplasmic membranes (13, 36). In contrast to observations of outer membrane damage, *E. coli* cells exposed for up to 120 h to acetic acid and high osmolarity (1.68 – 2.33 OsM) showed little loss of cytoplasmic membrane integrity, whether assessed by the combined PI and SYTO® 9 method, or using PI alone. Based on previous studies (9), ~ 2.0 $\log_{10}$ inactivation (i.e. 99%) of *E. coli* 2699 was expected after 120h for those pH and temperature conditions. Therefore, the results of the current study suggest either that only slight *E. coli* cytoplasmic membrane damage occurs under the conditions applied or, that rapid cell disintegration and destruction of nucleic acid polymers occurs following cytoplasmic membrane damage. A quantitative analysis by flow cytometry of total cell numbers could be used to address this ambiguity.

In contrast to PI, SYTO® 9 is regarded as a "membrane permeant" indicator, because of its ability to rapidly penetrate the intact cytoplasmic membrane (13, 36). Following entry to the cytoplasm, the indicator's fluorescence intensity is generally considered to increase only upon binding to DNA and RNA present in the cytoplasm (13, 36). However, during assessment of cytoplasmic membrane integrity by flow cytometry it was observed that SYTO® 9-fluorescence itself

increased strongly with increasing exposure time, in a similar manner to that observed for HI. Single factor analysis of variance indicated that the effect of osmolarity and NaCl concentration on SYTO® 9-staining was significant at all sample times after 2 h exposure. Significant ($p \leq 0.05$) non-monotonic staining of *E. coli* by SYTO® 9 in response to increasing osmolarity was observed at 24 h exposure, independent of the presence of sucrose.

Others have suggested that the outer membrane of Gram-negative bacteria may inhibit entry of SYTO® 9, and that differences in SYTO® 9-staining could be used as a measure of outer membrane damage in Gram-negative bacteria (5). Such increased SYTO® 9-fluorescence has been observed to coincide with a narrower distribution of SYTO® 9-staining, which has been presumed to arise from dye penetration being less affected by the distribution of permeabilities of the outer membrane (5). In this study, increases in HI- and SYTO® 9-fluorescence also coincided with observation of narrower distributions of fluorescence intensity evident in histograms derived by flow cytometry. However, staining location revealed by 3D-SIM suggests an additional hypothesis for increased, but less variable, HI- and SYTO® 9-fluorescence intensity.

As previously noted, it is normally assumed that increased SYTO® 9- and HI-fluorescence emission is only substantially increased on interaction with nucleic acids (DNA, RNA) (13). However, 3D-SIM showed bright SYTO® 9- and HI-stained regions in the plane of the cytoplasmic membrane. While often initially

localised in a few discrete domains, bright staining regions appeared to increase and spread with increased exposure time (and in response to changes in some other factors) resulting in more homogenous cytoplasmic membrane staining. In flow cytometry, the signal over the whole cell is measured as the cells pass through the excitation light beam (17). Thus, cells in which staining occurs in discrete regions against a relatively dark background will have a higher standard deviation than cells in which the fluorescent staining is more evenly distributed over the cell (17). Therefore another explanation for the development of populations with increased, but more homogenous SYTO® 9- and HI-fluorescence as assessed by flow cytometry is a change in cytoplasmic membrane composition that occurs with increasing frequency across the cell. The cationic nature of SYTO® 9 and HI that facilitates their interaction with anionic nucleic acid polymers (13), and it was hypothesised that the brightly stained cytoplasmic membrane domains may be rich in the strongly anionic (23) phospholipid cardiolipin.

The abundance of the major cytoplasmic membrane phospholipids in exponential phase *E. coli* cells are typically reported as 75% phosphatidylethanolamine, 20% phosphatidylglycerol, and 5% cardiolipin (11). It is known, however, that cardiolipin synthesis in *E. coli* increases dramatically during stationary phase (16). It has also been shown that cardiolipin-rich cytoplasmic membrane domains are predominantly located at the polar and septal regions in *E. coli* cells (25), and that cardiolipin-enrichment of the *E. coli* cytoplasmic membrane occurs in

response to osmotic stress (31). Our observations of discrete, brightly stained cytoplasmic membrane domains that are initially predominantly polar and that increase in frequency across the cell are, thus, consistent with cardiolipin enrichment. Using the cardiolipin-specific (25) dye NAO, a similar pattern of brightly stained cytoplasmic membrane domains was observed supporting the hypothesis that cardiolipin enrichment is associated with the increases in SYTO® 9- and HI-staining observed in response to exposure time. SYTO® 9- and HI-staining trends suggest that cardiolipin enrichment occurs most rapidly under conditions of high (initially hypertonic) and low (initially hypotonic) osmotic stress, and is comparatively suppressed at intermediate (isotonic, mildly hypertonic) osmolarities.

Based on the results of SYTO® 9-staining, cardiolipin enrichment also appeared to be suppressed by storage at 5°C. As assessed by HI staining, outer membrane damage appeared reduced at 5°C, but was not significantly reduced as assessed by crystal violet except after 120h exposure. These results reinforce the hypothesis that interpretation of HI-staining may be confounded by cell changes beyond those directly related to outer membrane permeability.

HI-staining was increased by pH reduction from pH 3.8 to pH 3.6 for all conditions assessed. In general, sensitisation to crystal violet also increased as pH decreased (i.e. acetic acid concentration increased). These observations are consistent with the hypothesis that acetic acid itself damages the outer

membrane. This conclusion is further supported by decreased outer membrane damage at neutral pH compared with that observed under acidic pH conditions, for several treatments involving high osmolarity. Cardiolipin enrichment as determined by SYTO® 9-staining was also increased by pH reduction from pH 4.0 to pH 3.8.

Apart from cardiolipin enrichment it also appeared that the cytoplasmic membrane was often plasmolysed to an extent from which the cell was unable to recover, at least while it remained under the treatment conditions. It is possible that the observed plasmolysis may be an artefact of the process of preparation for 3D-SIM, and particularly the mounting of samples in Vectashield®, which is a glycerol-based aqueous mounting medium. However, as glycerol is a membrane permeant solute, the rate of recovery from an osmotic upshift generated by glycerol is many times faster than that of the membrane impermeant solutes NaCl and sucrose (2). Therefore, it is more likely that the observed plasmolysis is a phenomenon caused by sustained exposure to NaCl and sucrose in a similar manner similar to that previously described (19) under other non-growth conditions.

In conclusion, the results of the present study demonstrate that prolonged exposure to acetic acid results in significant changes in both the outer and cytoplasmic membrane of *E. coli*. Under the range of conditions tested, outer membrane permeability increased in response to exposure time, and generally

more rapidly in response to both (initially) hypo- and hypertonic conditions, but cytoplasmic membrane damage *per se* did not. It therefore appears unlikely that the non-monotonic nature of the *E. coli* inactivation response to osmolytes in the presence of acetic acid is related to the integrity of the cytoplasmic membrane. However, cardiolipin-enrichment of the cytoplasmic membrane, as suggested by SYTO® 9 staining and flow cytometry and confirmed by NAO staining and 3D-SIM, *did* parallel the non-monotonic inactivation response of *E. coli* to increasing osmolarity in the presence of acetic acid previously reported (8, 9). Cardiolipin-enrichment and sustained plasmolysis of the cytoplasmic membrane are worthy of further investigation with respect to the potential mechanisms by which these changes might affect *E. coli* inactivation in the presence of acetic acid.

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TABLE 1. Nutrient broth (NB) formulations used in experiments

Formulation designation	Osmolarity (OsM)	NaCl % [a]	sucrose %	Initial expected tonicity [b]
J	0.17	0.5	0	hypotonic
A	0.34	1	0	isotonic
AS	0.34	0.5	6.2	isotonic
I	0.43	0.5	10	hypertonic
B	0.68	2	0	hypertonic
BS	0.68	0.5	21	hypertonic
C	1.02	3	0	hypertonic
H	1.21	3	10	hypertonic
D	1.34	4	0	hypertonic
E	1.68	5	0	hypertonic
F	1.99	6	0	hypertonic
G	2.33	7	0	hypertonic

[a] All concentrations are % wt/wt-on-water (i.e. g solute per 100g of water used to prepare broth, and include 0.5% NaCl present in NB base; [b] In minimal medium the growth rate of *E. coli* has been found to be maximal at ~0.3 OsM (external) (30) and this is assumed to represent isotonicity.

TABLE 2. Significance[a] (two-factor analysis of variance) of time and osmolytes (various factors) on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO®9

Indicator of membrane damage	Factor 2	Significance		Average % cells for Formulation / Osmolarity (OsM)												Average % cells at time (h)				
				J	A	AS	I	B	BS	C	H	D	E	F	G					
		Time (F1)	F2	0.17	0.34	0.34	0.43	0.68	0.68	1.02	1.21	1.34	1.68	1.99	2.33	2	24	48	72	120
crystal violet	[NaCl]	S[b]	S	79	60	51	39	45	44	58	74	79	91	99	99	42	79	85	85	90
	OsM 1	S	S													41	74	80	81	85
	OsM 2	S	S													42	71	80	78	84
	[sucrose]	S	S													23	45	62	58	69
	Solute A(S)	S	S													7	43	71	71	86
	Solute B(S)	S	NS[c]													9	50	54	47	64
HI	[NaCl]	S	S	45	44	37	42	47	27	47	40	43	43	47	56	2	9	39	84	99
	OsM 1	S	S													2	8	37	82	99
	OsM 2	S	S													2	8	30	76	98
	[sucrose]	S	S													1	4	21	61	95
	Solute A(S)	S	NS													1	3	35	68	97
	Solute B(S)	S	S													1	3	31	54	94
PI	[NaCl]	S	S	1	2	2	2	1	1	1	1	1	2	2	3	0	0	1	1	4
	OsM 1	S	S													0	1	1	1	4
	OsM 2	S	S													0	1	1	1	4
	[sucrose]	S	S													0	1	1	1	3
	Solute A(S)	S	NS													0	1	1	1	6
	Solute B(S)	S	NS													0	0	1	0	3
SYTO®9	[NaCl]	S	S	74	63	65	59	60	58	61	61	61	60	64	70	1	37	85	98	100
	OsM 1	S	S													1	33	85	98	100
	OsM 2	S	S													1	33	85	97	100
	[sucrose]	S	S													1	21	85	97	99
	Solute A(S)	S	NS													1	32	87	98	100
	Solute B(S)	S	S													1	17	83	96	99

[a] Compared on the basis of average percentage of cells displaying sensitivity to crystal violet, or PI or SYTO® 9 fluorescence intensity > 100 units, or HI fluorescence intensity > 38 units; [b] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S3)); [c] Not significantly different ($p > 0.05$);.

TABLE 3. Significance[a] (single factor analysis of variance) of NaCl concentration and osmolarity on *E. coli* membrane damage at different sample times, assessed by crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Indicator of membrane damage	Analysis abbreviation	Degrees of freedom	Significance at sample time (h)				
			2	24	48	72	120
crystal violet	[NaCl]	7	S[b]	S	S	S	S
	OsM 1	9	S	S	S	S	S
HI	[NaCl]	7	NS[c]	S	NS	NS	NS
	OsM 1	9	NS	S	NS	NS	NS
PI	[NaCl]	7	S	S	S	S	NS
	OsM 1	9	S	S	S	S	NS
SYTO® 9	[NaCl]	7	NS	S	S	S	S
	OsM 1	9	NS	S	S	S	S

[a] Compared on the basis of average percentage of cells displaying sensitivity to crystal violet, or PI or SYTO® 9 fluorescence intensity > 100 units, or HI fluorescence intensity > 38 units; [b] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S4)); [c] Not significantly different ($p > 0.05$).

TABLE 4. Significance[a] (two-factor analysis of variance) of time and temperature on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO®9

Indicator of membrane damage	Analysis abbreviation	Significance		Average % cells at temperature (°C)	
		Time (F1)	Temp (F2)	23	5
crystal violet	Temp J	NS[b]	S[c]	92.2	75.3
	Temp A	NS	S	74.5	48.5
	Temp AS	S	S	61.1	26.6
	Temp C	NS	NS	69.4	65.0
	Temp E	NS	NS	99.0	92.2
	Temp G	NS	S	100	99.8
HI	Temp J	S	S	56.0	4.27
	Temp A	S	S	55.0	3.97
	Temp AS	S	S	46.2	4.12
	Temp C	S	S	58.7	7.06
	Temp E	S	S	53.6	20.0
	Temp G	S	S	68.2	26.7
PI	Temp J	S	NS	0.936	0.371
	Temp A	NS	NS	2.25	0.277
	Temp AS	NS	S	1.86	0.452
	Temp C	S	S	1.22	0.386
	Temp E	S	S	2.61	1.95
	Temp G	S	NS	2.69	1.85
SYTO®9	Temp J	S	S	91.8	15.5
	Temp A	S	S	77.9	7.40
	Temp AS	S	S	80.4	7.77
	Temp C	S	S	75.6	9.85
	Temp E	S	S	74.5	19.3
	Temp G	S	S	87.0	21.4

[a] Compared on the basis of average percentage of cells displaying SYTO® 9 fluorescence intensity > 100 units; [b] Not significantly different ($p>0.05$); [c] Significantly different ($p\leq0.05$; p-values are provided in the Supplemental material (Table S5)).

TABLE 5. Significance[a] (two-factor analysis of variance) of time and pH on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO®9

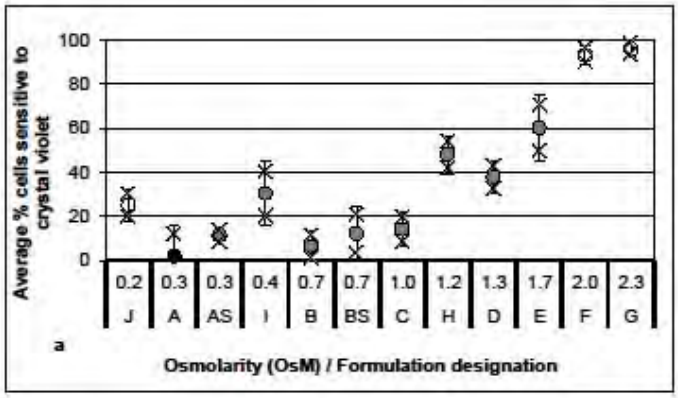
5

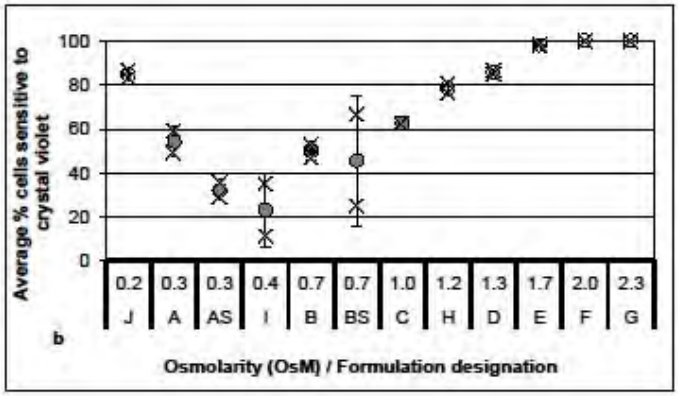
Indicator of membrane damage	Analysis abbreviation	Significance		Average % cells			Significance	
		Time (F1)	pH (F2)	pH 3.6	pH 3.8	pH 4.0	pH 3.6 vs. pH 3.8	pH 3.8 vs. pH 4.0
crystal violet	pH J	S[b]	S	92.7	75.3	42.3	S	S
	pH A	S	S	70.6	55.1	28.6	S	S
	pH B	S	S	59.8	39.9	22.8	NS	NS
	pH BS	S	S	60.3	47.0	30.9	S	S
	pH C	S	NS	65.0	55.6	49.3	n/a[d]	n/a
	pH E	S	S	97.0	89.3	83.2	S	NS
HI	pH J	S	S	53.6	32.0	33.8	S	NS
	pH A	S	S	55.4	30.9	34.2	S	NS
	pH B	S	S	56.9	34.3	32.0	S	NS
	pH BS	S	S	45.1	7.43	8.76	S	NS
	pH C	S	S	58.1	34.2	26.5	S	NS
	pH E	S	S	60.6	29.3	35.0	S	NS
PI	pH J	NS[c]	S	0.416	0.348	0.132	NS	S
	pH A	NS	S	0.142	0.580	0.107	S	S
	pH B	NS	S	0.119	0.494	0.080	S	S
	pH BS	NS	S	0.148	0.374	0.082	S	S
	pH C	S	S	0.360	0.576	0.072	NS	S
	pH E	S	S	0.171	1.25	0.194	S	S
SYTO®9	pH J	S	S	70.3	73.8	42.1	NS	S
	pH A	S	S	60.9	62.5	27.2	NS	S
	pH B	S	S	60.9	60.2	23.2	NS	S
	pH BS	S	S	61.5	58.2	12.5	NS	S
	pH C	S	S	61.1	60.1	17.4	NS	S
	pH E	S	S	63.6	59.8	34.9	NS	S

Membranes with acetic, time, osmolytes, cold & pH

[a] Compared on the basis of average percentage of cells displaying SYTO® 9 fluorescence intensity > 100 units; [b] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S6)); [c] Not significantly different ($p > 0.05$); [d] Not applicable.

Membranes with acetic, time, osmolytes, cold & pH





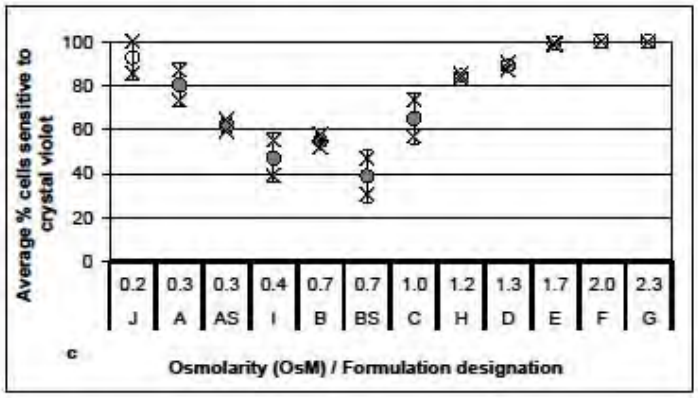


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FIG. 1. Average (circles) percentage of *E. coli* cells sensitive to crystal violet at different osmolarities in the presence of acetic acid at pH 3.8 after 2 (Fig. 1a), 24 (Fig. 1b) and 72 h (Fig. 1c) at 23°C. Black fill indicates formulation with minimum percentage injury, from among formulations containing NaCl only (i.e. excluding formulations AS, I, BS and H containing sucrose); grey fill indicates average percentage injury is not significantly different ($p>0.05$) to minimum percentage injury; no fill indicates average percentage injury is significantly different ($p\leq0.05$) to minimum percentage injury. Error bars indicate standard deviation (n = 2) and individual data for biological duplicates are shown (crosses).

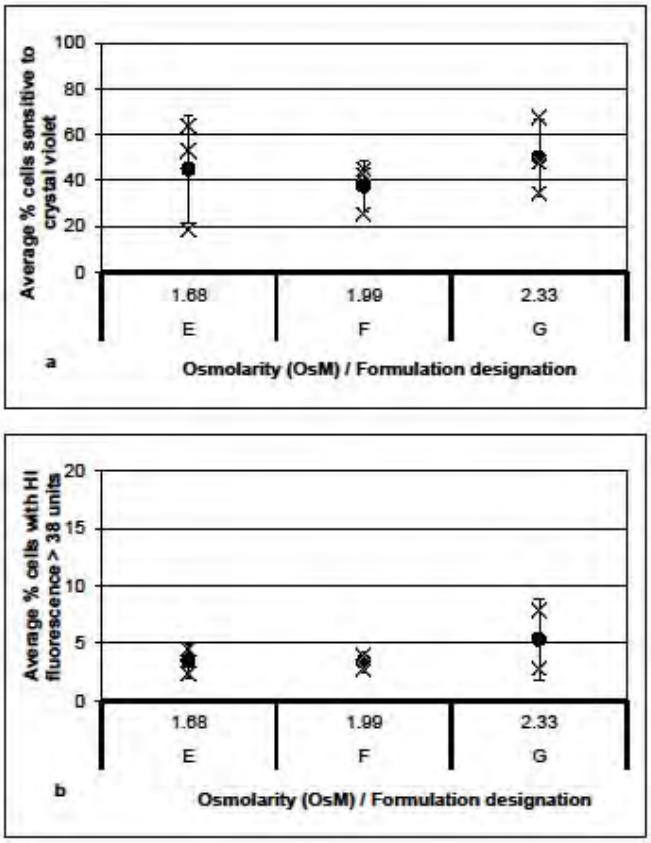


FIG. 2. Average percentage of cells sensitised to crystal violet (Fig. 2a) and average percentage of cells with HI fluorescence > 38 units (Fig. 2b) at neutral pH and high osmolarities after 2 h at 23°C. Error bars indicate standard deviation (Fig. 2a n = 3, Fig. 2b n = 2) and individual data for biological duplicates are shown (crosses).

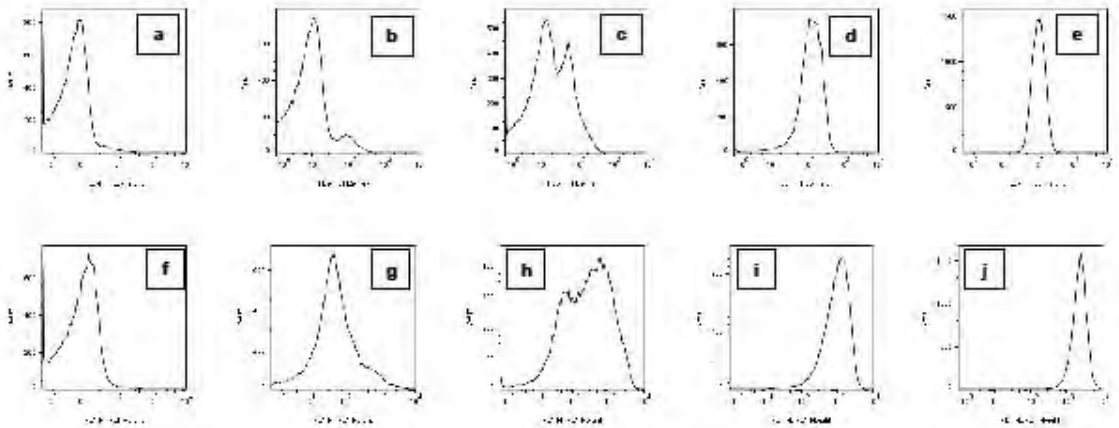
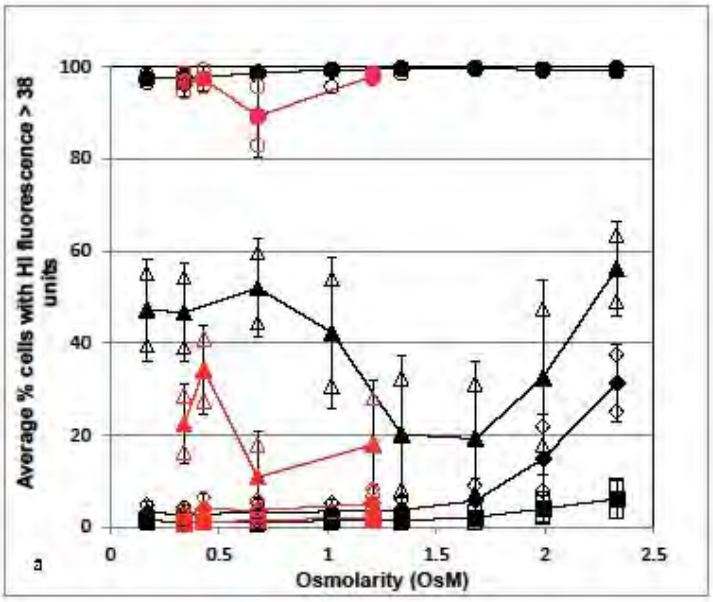


FIG. 3. Example fluorescence intensity histograms for hexidium iodide (FL2; Fig. 3a – 3e) and SYTO® 9 (FL1; Fig. 3f – 3j) staining of *E. coli* cells after exposure to Formulation E (1.68 OsM, pH 3.8) for 2 h (Fig. 3a and Fig. 3f), 24 h (Fig. 3b and Fig. 3g), 48 h (Fig. 3c and Fig. 3h), 72 h (Fig. 3d and Fig. 3i) and 120 h (Fig. 3e and Fig. 3j) at 23°C.


Average % cells with HI fluorescence > 38 units
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
a

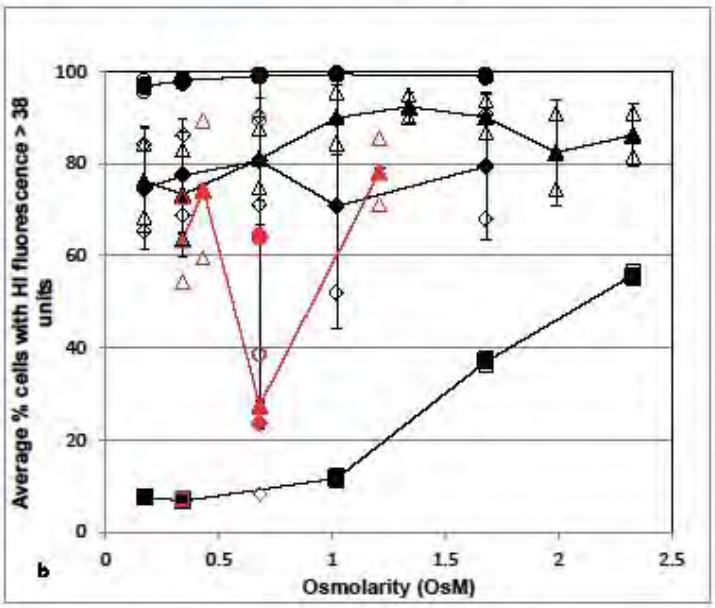

Average % cells with HI fluorescence > 38 units
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
b

FIG. 4. Average percentage of *E. coli* cells with hexidium iodide (HI) fluorescence > 38 units after exposure to acetic acid at pH 3.8 for 2 (squares), 24 (diamonds), 48 (triangles) or 120 h (circles) at 23°C (Fig. 4a). Average percentage of *E. coli* cells with hexidium iodide (HI) fluorescence > 38 units after exposure to acetic acid for 72 h at pH 3.8 at 5°C (squares), at pH 4.0 at 23°C (diamonds), at pH 3.8 at 23°C (triangles) or at pH 3.6 at 23°C (circles) (Fig. 4b). Black shapes indicate NB formulations containing NaCl only; red shapes indicate NB formulations containing sucrose and NaCl. Error bars indicate standard deviation (n = 2) and individual data for biological duplicates are shown (unfilled shapes).

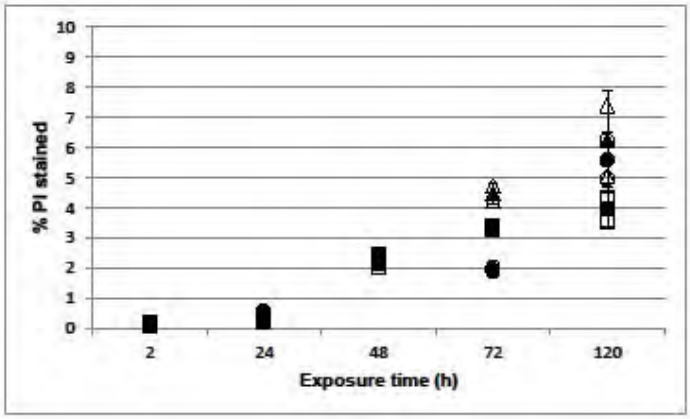
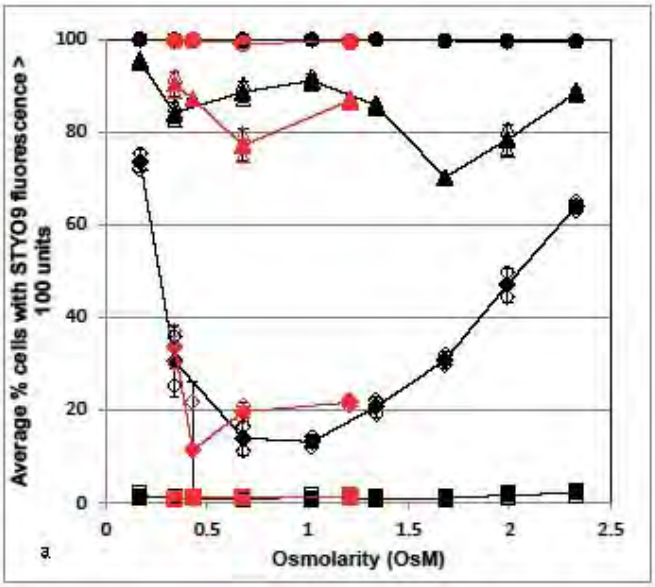


FIG. 5. Effect of osmolytes in the presence of acetic acid on temporal damage to *E. coli* assessed by propidium iodide (PI) (dual stain procedure). Circles = 1.68 OsM (Formulation E), squares = 1.99 OsM (Formulation F), triangles = 2.33 OsM (Formulation G) at 23°C. Error bars indicate standard deviation (n = 2) and individual data for biological duplicates are shown (unfilled shapes).


Average % cells with STYO9 fluorescence > 100 units
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
a

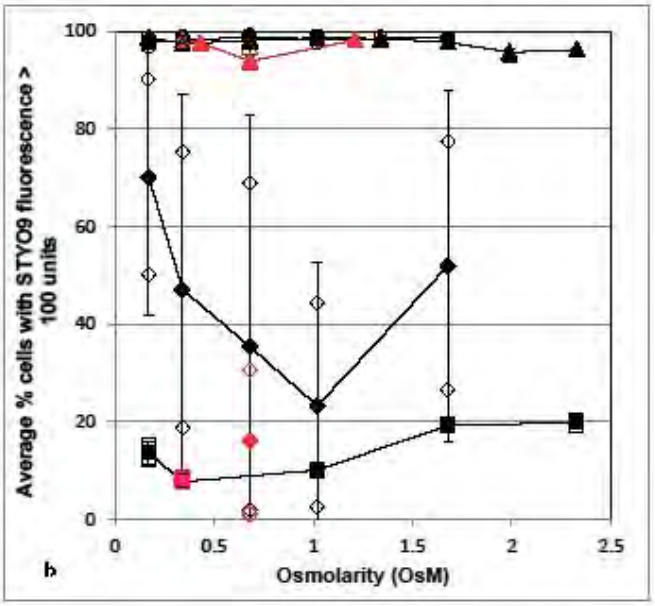

Average % cells with STYO9 fluorescence > 100 units
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
b

FIG. 6. Average percentage of *E. coli* cells with SYTO® 9 fluorescence > 100 units after exposure to acetic acid at pH 3.8 for 2 (squares), 24 (diamonds), 48 (triangles) or 120 h (circles) at 23°C versus calculated osmolarity (Fig. 6a). Average percentage of *E. coli* cells with SYTO® 9 fluorescence > 100 units after exposure to acetic acid for 72 h at pH 3.8 at 5°C (squares), at pH 4.0 at 23°C (diamonds), at pH 3.8 at 23°C (triangles) or at pH 3.6 at 23°C (circles) versus calculated osmolarity (Fig. 6b). Black shapes indicate NB formulations containing NaCl only; red shapes indicate NB formulations containing sucrose and NaCl. Error bars indicate standard deviation (n = 2) and individual data for biological duplicates are shown (unfilled shapes).

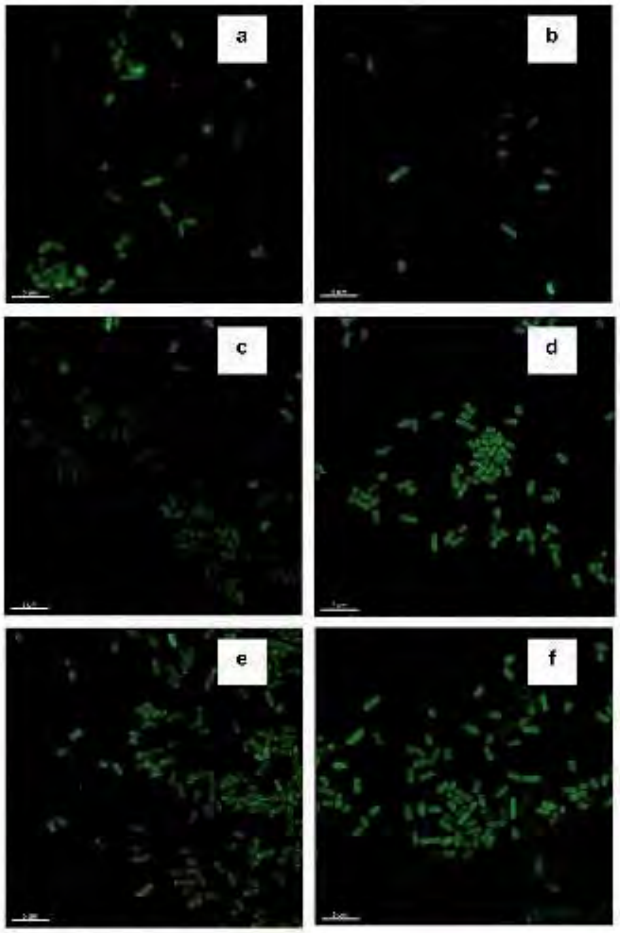


FIG. 7. Visualisation by 3D-SIM of SYTO® 9 staining of *E. coli* cells following exposure to Formulation J (0.34 OsM; top row), C (1.02 OsM; middle row) or G (2.33 OsM; bottom row) for 4 (Fig. 7a and 7e), 24 (Fig. 7b, 7c and 7f) or 72 (Fig. 7d) +/- 2 h. Scale bars = 5 µm.

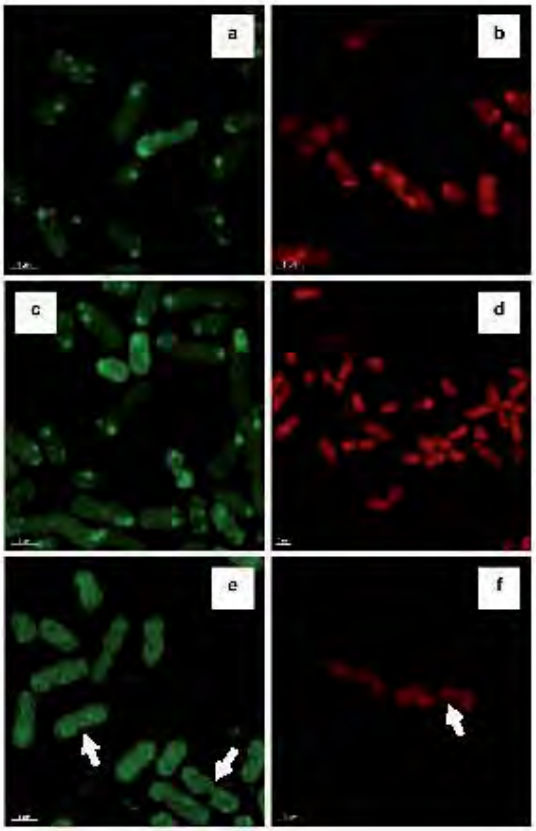


FIG. 8. Features revealed by 3D-SIM of SYTO® 9 (green) and hexidium iodide (HI, red) staining of *E. coli* cells exposed to (in order, Fig. 8a – 8f) Formulation G for 4 h, C for 4 h, G for 4 h, C for 72 h, G for 24 h and G for 48 h +/- 2 h. Bright staining of localised cytoplasmic membrane domains is emphasised in Fig. 8a – 8d. Delocalised staining and the occurrence of unstained plasmolysis spaces are emphasised in Fig. 8e and 8f (arrows). Scale bars = 1 µm.

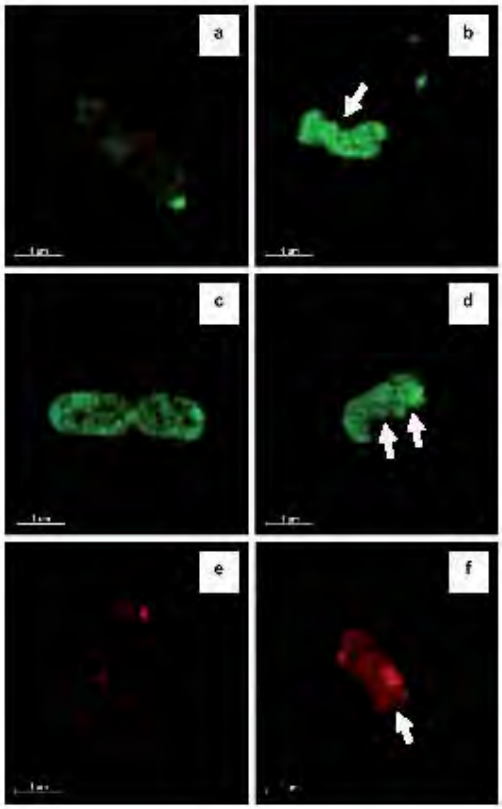


FIG. 9. Features revealed by 3D-SIM of nonyl acridine orange (NAO) staining of *E. coli* cells after 24 +/- 2 h exposure to (in order Fig. 9a – 9f) Formulations C, A, J, C, A and A. Green and red fluorescence is indicative of binding to cardiolipin. Bright staining of localised cytoplasmic membrane domains is emphasised in Fig. 9a, 9b and 9e. Delocalised staining is emphasised in Fig. 9b, 9c, 9d and 9f. Unstained areas presumed to represent plasmolysis spaces are observed in particular in Fig. 9b, 9d and 9f (arrows). Scale bars = 1 µm.

Supplemental material to -

Escherichia coli outer and cytoplasmic membrane changes during exposure to acetic acid, in response to exposure time, osmolytes, temperature and pH

B. Chapman, L. Turnbull, C. B. Whitchurch, T. Ross

Tables S1 – S6.

TABLE S1. Summary of two-factor ANOVA statistical analyses of *E. coli* membrane damage in response to exposure time and osmolytes, as assessed by crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Analysis abbreviation	Analysis description	Treatments (refer to Table 1) and exposure times included in analysis	Degrees of freedom			n[d]
			F1[a]	F2[b]	X[c]	
[NaCl]	Two-factor analysis of time and NaCl concentration	J, A, B, C, D, E, F and G all at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	7	28	40
OsM 1	Two-factor analysis of time and osmolarity, including sucrose containing formulations H and I	J, A, I, B, C, H, D, E, F and G all at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	9	36	50
OsM 2	Two-factor analysis of time and osmolarity as for OsM1 but replacing formulations A and B with AS and BS	J, AS, I, BS, C, H, D, E, F and G all at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	9	36	50
[sucrose]	Two-factor analysis of time and sucrose concentration	AS, I, BS and H all at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	3	12	20
Solute A(S)	Two-factor analysis of time and osmolyte type (NaCl vs. sucrose)	A and AS at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	1	4	10
Solute B(S)	Two-factor analysis of time and osmolyte type (NaCl vs. sucrose)	B and BS at pH 3.8 and 23°C at 2, 24, 48, 72 and 120 h	4	1	4	10

5

[a] Factor 1 (time) ; [b] Factor 2 (as per analysis description); [c] Interaction between Factor 1 and Factor 2; [d] Number of samples included in analysis.

TABLE S2. Summary of two-factor ANOVA statistical analyses of *E. coli* membrane damage in response to exposure time, storage temperature and pH, as assessed by crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO®9

Analysis abbreviation	Analysis description	Treatments (refer to Table 1) and exposure times included in analysis	Degrees of freedom			n[d]
			F1[a]	F2[b]	X[c]	
Temp J	Two-factor analysis of time and storage temperature	J at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h	3	1	3	8
Temp A		A at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h				
Temp AS		AS at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h				
Temp C		C at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h				
Temp E		E at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h				
Temp G		G at pH 3.8, 5°C and 23°C at 24, 48, 72 and 120 h				
pH J	Two-factor analysis of time and pH	J at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48 and 72 h	4	2	8	8
pH A		A at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48 and 72 h				
pH B		B at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48 and 72 h				
pH BS		BS at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48 and 72 h				
pH C		C at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48, 72 and 120 h				
pH E		E at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 2, 24, 48 and 72 h				

[a] Factor 1 (time) ; [b] Factor 2 (as per analysis description); [c] Interaction between Factor 1 and Factor 2; [d] Number of samples included in analysis.

TABLE S3. Significance[a] (two-factor analysis of variance) of time and osmolytes (various factors) on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Indicator or membrane damage	Factor 2	*p*-value		
		Time (F1)	F2	X[b]
crystal violet	[NaCl]	1.7E-22	2.9E-22	4.2E-8
	OsM 1	7.8E-24	3.9E-26	1.0E-7
	OsM 2	7.6E-21	4.1E-25	3.4E-6
	[sucrose]	1.5E-06	5.2E-06	0.049
	Solute A(S)	4.6E-07	0.031	NS
	Solute B(S)	1.4E-03	NS[c]	NS
HI	[NaCl]	1.0E-32	0.037	0.021
	OsM 1	2.8E-40	0.018	0.039
	OsM 2	7.0E-40	2.6E-07	3.8E-04
	[sucrose]	8.4E-16	1.6E-03	0.013
	Solute A(S)	1.8E-08	NS	NS
	Solute B(S)	6.7E-09	2.4E-05	2.4E-04
PI	[NaCl]	8.1E-14	3.6E-03	NS
	OsM 1	7.8E-17	2.3E-05	3.6E-03
	OsM 2	9.6E-23	4.0E-12	2.6E-07
	[sucrose]	4.0E-05	1.5E-05	0.035
	Solute A(S)	8.7E-03	NS	NS
	Solute B(S)	0.033	NS	NS
SYTO® 9	[NaCl]	1.4E-58	2.0E-21	9.7E-26
	OsM 1	2.4E-65	7.1E-18	3.6E-23
	OsM 2	2.4E-66	1.4E-19	1.0E-23
	[sucrose]	1.0E-23	5.7E-05	0.013
	Solute A(S)	1.2E-12	NS	NS
	Solute B(S)	7.2E-15	0.038	1.0E-03

[a] Compared on the basis of average proportion (%) of cells displaying sensitivity to crystal violet, or PI or SYTO® 9fluorescence intensity > 100 units, or HI fluorescence intensity > 38 units; [b] Interaction between Factor 1 and Factor 2; [c] Not statistically significantly different (p>0.05).

TABLE S4. Significance[a] (single factor analysis of variance) of NaCl concentration and osmolarity on *E. coli* membrane damage at different sample times, assessed by crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Indicator of membrane damage	Analysis abbreviation	Degrees of freedom	*p*-value at sample time (h)				
			2	24	48	72	120
crystal violet	[NaCl]	7	2.6E-05	6.0E-07	3.3E-03	8.4E-04	2.3E-05
	OsM 1	9	1.5E-05	2.2E-06	3.5E-04	1.0E-04	9.9E-05
HI	[NaCl]	7	NS[b]	6.8E-03	NS	NS	NS
	OsM 1	9	NS	4.3E-03	NS	NS	NS
PI	[NaCl]	7	0.011	0.042	0.018	9.2E-09	NS
	OsM 1	9	0.016	3.0E-06	1.6E-03	1.6E-10	NS
SYTO® 9	[NaCl]	7	NS	8.9E-07	1.4E-05	7.3E-05	0.046
	OsM 1	9	NS	4.8E-06	1.6E-06	1.2E-05	0.024

[a] Compared on the basis of average proportion (%) of cells displaying sensitivity to crystal violet, or PI or SYTO® 9 fluorescence intensity > 100 units, or HI fluorescence intensity > 38 units; [b] Not statistically significantly different (p>0.05).

TABLE S5. Significance[a] (two-factor analysis of variance) of time and temperature on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Indicator of membrane damage	Analysis abbreviation	*p*-value		
		Time (F1)	Temp (F2)	X[b]
crystal violet	Temp J	NS[c]	0.011	NS
	Temp A	NS	5.5E-03	NS
	Temp AS	1.0E-04	3.5E-06	0.049
	Temp C	NS	NS	NS
	Temp E	NS	NS	NS
	Temp G	NS	0.015	NS
HI	Temp J	7.4E-06	8.4E-08	2.8E-5
	Temp A	1.6E-05	1.9E-07	5.4E-05
	Temp AS	5.7E-06	4.2E-07	2.2E-05
	Temp C	6.9E-06	2.5E-07	6.6E-05
	Temp E	1.1E-06	7.9E-06	5.2E-04
	Temp G	7.8E-07	3.2E-07	0.016
PI	Temp J	0.032	NS	0.027
	Temp A	NS	NS	NS
	Temp AS	NS	0.018	0.049
	Temp C	5.4E-04	3.5E-04	1.5E-03
	Temp E	6.5E-06	4.0E-03	2.2E-04
	Temp G	0.012	NS	NS
SYTO® 9	Temp J	1.2E-03	7.7E-13	1.8E-06
	Temp A	6.0E-07	3.2E-11	3.0E-07
	Temp AS	4.5E-08	1.7E-12	3.2E-08
	Temp C	6.9E-10	5.1E-13	4.1E-10
	Temp E	1.3E-09	2.8E-13	2.8E-10
	Temp G	1.9E-04	1.8E-11	4.0E-06

[a] Compared on the basis of average proportion (%) of cells displaying SYTO® 9 fluorescence intensity > 100 units; [b] Interaction between Factor 1 and Factor 2; [c] Not statistically significantly different (p>0.05).

TABLE S6. Significance[a] (two-factor analysis of variance) of time and pH on *E. coli* membrane damage as assessed using crystal violet, hexidium iodide (HI), propidium iodide (PI) and SYTO® 9

Indicator of membrane damage	Analysis abbreviation	*p*-value			*p*-value	
		Time (F1)	pH (F2)	X[b]	pH 3.6 vs. pH 3.8	pH 3.8 vs. pH 4.0
crystal violet	pH J	3.0E-08	7.6E-09	3.2E-03	1.0E-05	2.7E-05
	pH A	1.5E-05	3.1E-04	NS	0.026	8.1E-03
	pH B	0.013	0.023	NS	NS	NS
	pH BS	5.7E-04	3.9E-04	0.028	3.3E-03	0.025
	pH C	4.6E-04	NS	NS	n/a	n/a
	pH E	1.3E-07	1.0E-03	3.8E-04	0.023	NS
HI	pH J	5.5E-10	1.6E-04	0.048	1.3E-04	NS
	pH A	1.6E-09	1.4E-04	0.035	8.1E-05	NS
	pH B	2.2E-08	8.7E-04	NS	1.2E-04	NS
	pH BS	4.7E-03	2.9E-04	NS	1.42E-03	NS
	pH C	8.9E-08	3.8E-04	0.019	1.9E-04	NS
	pH E	1.6E-08	5.9E-05	3.8E-03	3.4E-05	NS
PI	pH J	NS[c]	5.0E-06	0.025	NS	2.5E-05
	pH A	NS	4.0E-04	NS	4.7E-03	3.0E-03
	pH B	NS	4.2E-03	NS	0.022	0.013
	pH BS	NS	1.6E-04	NS	4.9E-03	1.1E-03
	pH C	0.022	5.5E-03	NS	NS	7.0E-03
	pH E	9.8E-11	5.5E-13	1.7E-10	1.5E-09	4.6E-10
SYTO® 9	pH J	4.2E-06	1.8E-03	NS	NS	2.2E-04
	pH A	7.7E-08	1.2E-04	NS	NS	1.2E-03
	pH B	1.2E-07	8.8E-05	0.025	NS	1.4E-03
	pH BS	1.5E-06	8.1E-06	0.027	NS	2.2E-04
	pH C	1.3E-06	6.2E-05	0.033	NS	1.1E-03
	pH E	2.9E-09	1.2E-04	0.020	NS	1.2E-03

[a] Compared on the basis of average proportion (%) of cells displaying SYTO® 9 fluorescence intensity > 100 units; [b] Interaction between Factor 1 and Factor 2; [c] Not statistically significantly different (p>0.05).

7 Manuscript 5: Cardiolipin in *E. coli* and *S. enterica* with acetic

Chapman, B. and T. Ross. Cardiolipin production by *Escherichia coli* and *Salmonella enterica* in response to time, osmolytes, temperature and pH during exposure to acetic acid, and changes in membrane potential and fluidity.

In preparation

Cardiolipin production by *Escherichia coli* and *Salmonella enterica* in response to time, osmolytes, temperature and pH during exposure to acetic acid, and changes in membrane potential and fluidity

Running title – Cardiolipin in *E. coli* and *S. enterica* with acetic

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KEYWORDS

E. coli; *Salmonella*; cardiolipin; acetic acid; NaCl; cold; pH; membrane potential; membrane fluidity

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The kinetics of cardiolipin production by *Escherichia coli* and *Salmonella enterica* during prolonged exposure to acetic acid were examined using nonyl acridine orange (NAO) and flow cytometry. For *E. coli* the proportion of cells exhibiting red-shifted NAO (rsNAO) fluorescence characteristic of interaction with cardiolipin was non-monotonic with respect to osmolarity overall and, at exposure times ≤ 24 h, had an inflection point close to isotonicity. For both microorganisms rsNAO fluorescence generally increased with increased exposure time, decreased pH, and storage at 5°C, and was greater in the presence of acetic compared with hydrochloric acid. Cardiolipin production occurred more rapidly in *S. enterica* than in *E. coli*. Cardiolipin enrichment maintained membrane potential in a larger percentage of cells. However, at shorter exposure times survival of *E. coli* cells producing cardiolipin at intermediate (initially hypertonic) osmolarities was disadvantaged by more rapid depolarisation. For both *E. coli* and *S. enterica*, fluorescence polarisation showed a clear overall decrease in membrane fluidity after 6 h, in line with the apparent overall increase in cardiolipin. However, a complex, kinetically shifting bimodal response of membrane fluidity to increasing osmolarity was noted. Changes in cardiolipin content in the presence of acetic acid were observed in response both to the acid itself, as well to other variables. From knowledge of cardiolipin structure and its roles in membranes, cardiolipin content is considered likely to be involved in the observed changes in membrane potential and fluidity.

Prolonged exposure to acetic acid results in significant changes in both the outer and cytoplasmic membranes of *Escherichia coli* (9). In the presence of acetic acid at pH 3.6 – 3.8 permeability to crystal violet and hexidium iodide, normally excluded by the outer membrane (21, 25), increased in response to exposure time (9). In addition, *E. coli* staining by the cytoplasmic membrane-permeant nucleic acid stain SYTO® 9 increased with increasing exposure time, as determined by flow cytometry (9). While others have suggested increases in SYTO® 9 staining may relate to changes in outer membrane permeability (5), Three Dimensional Structured Illumination Microscopy (3D-SIM) suggested the increases were at least in part related to changes in the cytoplasmic membrane (9). Using 3D-SIM, very bright SYTO® 9- staining of discrete domains located in the plane of the cytoplasmic membrane were revealed (9). It was hypothesised that the brightly stained cytoplasmic membrane domains could be rich in the strongly anionic (20) phospholipid cardiolipin. Studies undertaken using 3D-SIM and the fluorescent indicator 10-*N*-nonyl acridine orange (NAO), which has a high affinity for cardiolipin (23), revealed a very similar pattern of cytoplasmic membrane staining to that visualised using SYTO® 9 (9).

The inactivation response of *Escherichia coli* to acetic acid is non-monotonic under conditions of increasing osmolarity imposed by either NaCl or sucrose (6, 7) and has also been shown to be affected by pH / acetic acid concentration (6, 7, 8) and by storage temperature (8). Apart from an overall trend to increased SYTO® 9 fluorescence with increased exposure time to acetic acid after 24 h

exposure we observed (9) transient, non-monotonic (i.e. initially decreasing and then increasing) SYTO® 9 fluorescence with increasing osmolarity, irrespective of the osmolyte employed. Thus, the kinetics of presumptive cardiolipin-enrichment of the cytoplasmic membrane suggested by SYTO® 9 staining and flow cytometry (9) paralleled the previously reported non-monotonic inactivation response of *E. coli* to increasing osmolarity in the presence of acetic acid previously reported (6, 7). It was also noted that SYTO® 9 staining was decreased in cells stored at 5°C, compared with 23°C, and for cells exposed to pH 4.0 compared with pH 3.8 (9).

Cardiolipin is one of three major cytoplasmic membrane phospholipids present in *E. coli* (10). The phospholipid composition of *Salmonella* has been shown to be quite similar to that of *E. coli*, albeit with slightly decreased levels of anionic phospholipids, including cardiolipin, as a percentage of total phospholipids (1). Cardiolipin synthesis in *E. coli* is known to increase dramatically during stationary phase (13) and in response to osmotic stress (35), irrespective of the nature of the osmolyte (NaCl or sucrose) (42). Cardiolipin production under hypotonic conditions is thought to improve functioning of the mechanosensitive cytoplasmic solute channel MscS, with which it is co-located, and which is responsible for export of excess osmolytes from the cell cytoplasm (33). Under hypertonic conditions cardiolipin improves functioning of the co-located H^+-osmolyte symporter ProP, which is responsible for the inward transport of organic compatible solutes (35, 42). Cardiolipin enrichment under hypertonic conditions

may also serve as a source of the compatible solute glycerol (34), which is produced on condensation of two phosphatidlyglycerol molecules to form one cardiolipin molecule (38).

Due to their relatively small headgroup size, polar lipids such as cardiolipin tend to increase ordering of their fatty acid acyl chains (20, 44), forming tightly packed domains with reduced membrane fluidity (31). However, the onset of domain boundary instabilities between, for example, areas rich and poor in cardiolipin may actually increase overall membrane fluidity (26). Weak acid theory does not consider the influence of cytoplasmic membrane changes on acetic acid permeability, despite observation in other membrane systems of the sensitivity of acetic acid permeability to membrane chain ordering (46). However others have suggested that alteration of the phospholipid content of the cytoplasmic membrane could result in altered resistance to weak acids (22) via changes in proton or anion permeability, or both (2). It was therefore hypothesised that changes in cardiolipin content under hypotonic and hypertonic conditions could contribute towards an explanation for the observed non-monotonic nature of the *E. coli* inactivation (6, 7).

The primary aim of this study was to determine the effects of time and osmolarity on *E. coli* and *S. enterica* cardiolipin production in the presence of acetic acid. Cardiolipin production was assessed using the fluorescent indicator 10-*N*-nonyl acridine orange (NAO) (23) and flow cytometry. In addition to demonstrating high

affinity for cardiolipin, accumulation of NAO is affected by membrane potential changes (17, 18). Once driven into cells by membrane potential gradients and lipophilicity, NAO is locked into cardiolipin-containing membranes by its (tracking dye-like) nonyl side chain (40). Membrane potential changes coinciding with changes in cardiolipin content were also assessed using NAO. Membrane fluidity changes coincident with changes in cardiolipin content were assessed using fluorescence polarisation of diphenylhexatriene (DPH) and trimethylammonium-diphenylhexatriene (TMA-DPH), which target the acyl chain and headgroup regions, respectively, of the cytoplasmic membrane (16, 30). The comparative effects of storage temperature and pH on cardiolipin production and membrane fluidity were briefly assessed, to confirm suggestions (9) based on SYTO® 9-staining that cardiolipin production may be decreased at lower storage temperatures, and increased at lower pH. The effect of acidulent type was also considered to determine whether cardiolipin enrichment was favoured in the presence of acetic acid, a 'weak' acid, compared with the HCl, a fully dissociable ('strong') acid.

MATERIALS AND METHODS

Bacterial cultures and inoculum preparation. Non-pathogenic *E. coli* strain FRRB (Food Research Ryde Bacterial culture collection) 2699, and *S. enterica* FRRB 2742 (*S.* Montevideo) were maintained as glycerol stocks at -80°C. Inocula for experiments were prepared by transferring cells from glycerol stocks

into 10 ml of NB incubated for 22 h (+/- 1 h) at 37°C (+/-1°C). Aliquots, 10 µl, of the 22 h NB cultures were transferred to 10 ml tryptone soy broth (TSB; Oxoid, CM0129) containing 1% total glucose (TSB1%G), and incubated with shaking at 200 rpm for 22 h (+/- 1 h) at 37°C (+/-1°C). At the conclusion of incubation, the pH of TSB1%G inocula were determined using pH indicator papers (Type CS, pH 3.8 – 5.5; Whatman International Ltd, UK) to ensure that it was approximately 4.2 (+/- 0.1), indicating that acid conditioning of the cells had occurred.

Experimental treatments. Cardiolipin production and membrane potential and fluidity in response to osmolarity and osmolyte type were assessed in ten acidified Nutrient Broth (NB; CM001; Oxoid, UK) formulations (Table 1) sampled after exposure times of 6, 24, 48 and 72 h (+/- 1 h). Eight formulations (J, A, B, C, D, E, F, G) contained NaCl (Sigma Chemical Co., USA) only, and two formulations (AS, H) contained sucrose (food grade; CSR Sugar, Australia) and NaCl (Table 1). Calculated osmolarities for the twelve treatments were between 0.17 and 2.33 OsM (Table 1); contributions to osmolarity by acids and components of the NB base apart from NaCl (0.5% w/v) were ignored in osmolarity calculations. The treatment pair A and AS, were formulated to achieve the same calculated osmolarity, arising from either NaCl (A) or predominantly from sucrose (AS) (Table 1). Formulations were acidified with glacial acetic acid (Sigma Chemical Co., USA), and the pH adjusted to pH 3.8 or pH 4.0, for *E. coli* and *S. enterica* respectively, using a Beckman model 390 pH meter with probe 511080 (Beckman Coulter, Inc., USA). NB formulations were filter-sterilized (0.22

μm; Millipore, USA), and aseptically dispensed into sterile 28 ml screw-capped polypropylene containers and equilibrated overnight to 23°C (+/-1°C) prior to inoculation for experiments.

In addition to the effects of osmolarity and osmolyte type, the effects of pH, acidulent type, and cold storage on cardiolipin production and membrane potential and fluidity were also assessed. For *E. coli*, four additional treatments were prepared; Formulation A (Table 1; isotonic 0.8 MPa) acidified with glacial acetic acid to pH 3.6 and 4.0, and to pH 3.6 with concentrated hydrochloric acid (HCl; Sigma Chemical Co., USA), and Formulation C (Table 1) stored at 5°C. For *S. enterica*, two additional treatments were prepared; Formulation A (Table 1; isotonic 0.8 MPa) acidified with concentrated hydrochloric acid (HCl; Sigma Chemical Co., USA) to pH 3.6 (previously determined as the growth / no growth interface for *S. enterica* (7)), and Formulation C (Table 1) stored at 5°C.

Treatment inoculation and incubation. For all experiments triplicate, individually grown, TSB1%G cultures were used to inoculate 20 ml lots of NB formulations to an initial cell concentration of ~10^7 CFU/g. Inoculated treatment broths were incubated statically at 23°C (+/-1°C), except for cold storage experiments, for which inoculated samples were gradually cooled from 23°C to 5°C (+/-1°C) over a period of 2 h in a programmable incubator (APT.line® KB incubator; Binder Inc., USA), and then maintained at 5°C (+/-1°C) for the remainder of the experiment. Samples from 5°C treatments were allowed to

equilibrate for 30 min in a 23°C water bath prior to staining for fluorescence based assessments.

Flow cytometric assay of cardiolipin production and membrane potential. Cardiolipin production and depolarisation was assessed using NAO (Molecular Probes, Invitrogen, USA). At each sampling time, 0.5 ml aliquots of inoculated treatments were aseptically withdrawn and mixed with 0.5 µL of NAO solution (200 µM NAO in dimethyl sulfoxide (DMSO; Sigma, USA) to give a final concentration of 200 nM NAO), and incubated in the dark at room temperature (approx. 23°C) for 1 h (23) prior to flow cytometric analysis.

Flow cytometry for NAO staining was performed using a Becton Dickinson FACSAria™ flow cytometer equipped with a 15mW 488 nm air-cooled argon laser for excitation of the fluorescent dyes. Osmosol (Lab Aids Pty Ltd, Sydney, Australia) (180 meq/L sodium, 153 meq/L chloride, 5.1 meq/L potassium, 1.0 meq/L EDTA) was used as the sheath fluid for all experiments. Events were collected at a low flow rate corresponding to ≤ 1000 events / s, and 50,000 events were collected in each analysis. Forward scatter (FSC), side scatter (SSC) and fluorescence measurements were recorded for each event, logarithmically amplified, and converted into digital signals for further analysis using FlowJo software (Tree Star, Inc., Oregon, USA) software.

Event populations representing bacteria were discriminated and gated in bivariate intensity plots of FSC versus SSC. The use of Osmosol resulted in a refractive index mismatch between the sheath fluid and the sample for some formulations but, by allowing for an increase in the FSC signal with increasing osmolarity bacterial cells were easily discriminated from background noise. Following FSC versus SSC gating, subpopulations with and without elevated amounts of cardiolipin were discriminated on the basis of their bivariate green (502 long pass mirror and 530/30 nm band pass filter) and red fluorescence (595 long pass mirror and 610/20 band pass filter). Green fluorescence results from association of NAO with anionic phospholipids (phosphatidylglycerol and cardiolipin), but red-shifted NAO- (rsNAO-) fluorescence results only from association of NAO with cardiolipin (23).

Four distinct subpopulations were discriminated, with high red / high green, high red / low green, low red / high green, and low red / low green fluorescence combinations. The membrane potential states of the four subpopulations were attributed as depolarised, polarised, polarised and depolarised, respectively, based on the results of experiments with *E. coli* examining the effect of depolarisation by carbonylcyanide *m*-chlorophenylhydrazone (CCCP) on NAO green and red fluorescence intensity (data not shown). The addition of CCCP did not affect red fluorescence. However, cells with high levels of red fluorescence (elevated cardiolipin) and low levels of green fluorescence responded to depolarisation by CCCP with an increase in green fluorescence, while cells

initially displaying only high green fluorescence responded with a decrease in green fluorescence. Thus the proportion of depolarised cells was able to be determined for populations with and without elevated cardiolipin (rsNAO fluorescence).

Fluorescence polarisation assay of membrane fluidity. Membrane fluidity was determined by fluorescence polarisation of the probes DPH and TMA-DPH (both Sigma, USA). Treatments were inoculated and incubated as described above, using a staggered inoculation approach to synchronise all sample times. Aliquots (100 µL) of each treatment and representing each sampling time were aseptically withdrawn to 96-well microtitre plates and 1 µL aliquots of DPH (10^{-6} M DPH in tetrahydrofuran) or TMA-DPH (10^{-6} M TMA-DPH in dimethylsufoxide) solution added. Samples were incubated at 23°C for 1 h with shaking at 100rpm prior to measurement of fluorescence polarisation using a POLARstar Omega microplate reader equipped with UV fluorescence polarisation optics (BMG Labtech Inc., NC, USA), a 355 nm excitation filter and two identical 430 nm emission filters positioned 180° apart. Data were acquired in endpoint mode. The positioning delay was set at 1 s, with 50 flashes per well. The target mP was set at 100. For each biological replicate an automatic gain adjustment was performed on the 24 h Formulation G (highest osmolarity) sample, just prior to assay commencement.

Statistical analysis. Statistical comparisons of the effects of exposure time and osmolytes, storage temperature, pH and acidulent type on cardiolipin production, membrane potential and fluidity were made by two-factor analysis of variance ($p \leq 0.05$) using the ANOVA: Two-factor with replication tool of Microsoft® Excel. Analyses applied are summarised in the Supplemental material (Table S1). For the effect of osmolytes, comparisons were made on the basis of the concentration of NaCl, osmolarity (including both NaCl only- and sucrose-containing formulations) and solute type (comparing formulation A with AS). Statistical comparisons of the effects of NaCl concentration and osmolarity on cardiolipin production, membrane potential and fluidity at each exposure time were also made by single factor analysis of variance ($p \leq 0.05$) using the single factor ANOVA tool of Microsoft® Excel.

RESULTS

Cardiolipin production in response to time and osmolytes. The effects of time and osmolytes on cardiolipin production for *E. coli* are shown in Table 2. The effects on *S. enterica* are shown in Table 3. Time and NaCl concentration and osmolarity had significant ($p \leq 0.05$) effects on cardiolipin production for both microorganisms.

For *E. coli* the effect of both time and osmolarity on cardiolipin production was non-monotonic overall when analysed by two factor analysis of variance (Table

2). The percentage of *E. coli* cells with rsNAO-fluorescence was higher after 6 h exposure than after 24 h exposure, but then increased again after 48 and 72 h exposure (Table 2 and Figure 1a). Overall, the minimum percentage of *E. coli* cells with rsNAO-fluorescence occurred in formulations around isotonicity (Table 2). Single factor analysis of variance showed a statistically significant ($p \leq 0.05$) effect of both NaCl concentration and osmolarity on the percentage of *E. coli* cells with rsNAO-fluorescence after 6 and 24 h exposure (Table 4), shown in Figure 1a.

In comparison with *E. coli*, the percentage of *S. enterica* cells with rsNAO-fluorescence increased in a statistically significant ($p \leq 0.05$) monotonic fashion with increasing osmolarity (Table 3). Around isotonicity (i.e. Formulation A, 0.34 OsM), the percentage of *S. enterica* cells with rsNAO-fluorescence was noticeably greater than the percentage of *E. coli* cells with rsNAO-fluorescence after 6 and 24 h exposure (Figure 1). As for *E. coli*, single factor analysis of variance showed a statistically significant ($p \leq 0.05$) effect of both NaCl concentration and osmolarity on the percentage of *S. enterica* cells with rsNAO-fluorescence after 6 and 24 h exposure (Table 4), shown in Figure 1b. Unlike *E. coli* (Table 2), the percentage of rsNAO-stained *S. enterica* cells was significantly ($p \leq 0.05$) decreased in the presence of sucrose at 0.34 OsM (i.e. paired formulations A and AS) (Table 3).

Cardiolipin production in response to temperature, pH and acidulent. The effects of time and temperature, pH and acidulent on cardiolipin production and membrane fluidity are shown in Tables 5, 6 and 7, respectively. The percentage of both *E. coli* and *S. enterica* cells with rsNAO-fluorescence was increased significantly ($p \leq 0.05$) at 5°C compared with 23°C (Table 5). For *E. coli*, the percentage of cells with rsNAO-fluorescence was also statistically significantly decreased in the presence of acetic acid at pH 4.0 compared with pH 3.8 (Table 6). At pH 3.6, significantly ($p \leq 0.05$) fewer *E. coli* cells displayed rsNAO-fluorescence in the presence of hydrochloric, compared with in the presence of acetic acid (Table 7). For *S. enterica*, the percentage of cells with rsNAO-fluorescence was less in the presence of acetic acid at pH 4.0, compared with in the presence of hydrochloric acid at pH 3.6 (Table 7).

Membrane potential in response to time and osmolytes. In *E. coli*, depolarisation significantly ($p \leq 0.05$) increased with increasing exposure time, both in populations with and without elevated cardiolipin (rsNAO) (Table 2 and Figure 2). Among *E. coli* cells displaying rsNAO, the effect of increasing NaCl concentration / osmolarity was difficult to interpret (Tables 2 and 4), with no clear trend in depolarisation apparent. However, among *E. coli* cells not displaying rsNAO-fluorescence, there was a significant ($p \leq 0.05$) effect of increasing NaCl concentration / osmolarity on depolarisation (Tables 2 and 4). A complex response was observed, with depolarisation initially increasing, then decreasing, and then increasing again with increasing osmolarity (Figure 2).

Among *S. enterica* cells displaying rsNAO-fluorescence, there was no clear trend of depolarisation in response to time (Tables 3 and 4). Irrespective of the treatment conditions > 50% of *S. enterica* cells with rsNAO appeared depolarised at the first sampling time at 6 h (Figure 3a). In comparison, among *S. enterica* cells not displaying rsNAO, there was a significant effect of increasing osmolarity on depolarisation (Table 3) at all sample times (Table 4). At sample times ≥ 24 h, depolarisation increased monotonically with increasing osmolarity (Figure 3). However at 6 h, the trend in depolarisation with increasing osmolarity mirrored the more complex increasing / decreasing / increasing pattern observed for *E. coli* (compare Figure 3a and Figure 2a). Depolarisation among cells not displaying rsNAO was significantly ($p \leq 0.05$) reduced in the presence of sucrose compared with NaCl for *S. enterica* (Table 3), but not for *E. coli* (Table 2).

Membrane potential in response to temperature, pH and acidulent. Depolarisation was significantly ($p \leq 0.05$) greater at 5°C than at 23°C for *E. coli* cells with rsNAO-fluorescence, but significantly reduced for cells without rsNAO-fluorescence (Table 5). Storage temperature did not have a significant effect on depolarisation of *S. enterica* (Table 5). *E. coli* depolarisation significantly decreased with increasing pH (Table 6) and, in the presence of HCl compared with acetic acid at pH 3.6 (Table 7). Among *S. enterica* cells not displaying rsNAO, depolarisation was significantly ($p \leq 0.05$) reduced in the presence of HCl at pH 3.6, compared with acetic acid at pH 4.0.

Membrane fluidity in response to time and osmolytes. The responses determined by DPH and TMA-DPH were generally similar. Exposure time had a significant ($p \leq 0.05$) effect on polarisation of both DPH and TMA-DPH for *E. coli* (Table 2) and for *S. enterica* (Table 3), with an overall decrease in membrane fluidity (increase in polarisation) with increasing exposure time (Figures 4 and 5). Osmolarity also significantly ($p \leq 0.05$) affected polarisation of both probes for both microorganisms (Tables 3 and 4), at most sample times (Table 4). The effect of increasing osmolarity on membrane fluidity was complex, tending towards a bimodal response at most samples times (Figures 4 and 5) which was not correlated with cardiolipin production (i.e. rsNAO-fluorescence). In the presence of sucrose, *E. coli* membrane fluidity was significantly increased (i.e. polarisation was significantly decreased), compared with in the presence of NaCl (Table 2). However, osmolyte type did not significantly affect *S. enterica* membrane fluidity (Table 3).

Membrane fluidity in response to temperature, pH and acidulent. Cold storage significantly ($p \leq 0.05$) decreased membrane fluidity (increased polarisation) in both the head group and lipid tail regions of *E. coli* membranes, but did not affect *S. enterica* membrane fluidity (Table 5). *E. coli* membrane fluidity was also decreased at pH 3.8 compared to pH 3.6 (Table 6), but was not significantly different in the presence of HCl versus acetic acid (Table 7). *S. enterica* membrane fluidity in the head group region only was significantly

($p \leq 0.05$) increased in the presence of HCl at pH 3.6, compared to acetic acid at pH 4.0 (Table 7).

DISCUSSION

It was hypothesised that changes in cardiolipin content under hypotonic and hypertonic conditions could contribute towards an explanation of the non-monotonic response of *E. coli* inactivation to osmolarity in the presence of acetic acid previously described (6, 7). The results presented here show that cardiolipin production in *E. coli* is also non-monotonic, with an inflection point close to isotonicity. The inflection point in the inactivation response to increasing osmolarity was previously determined to occur at "intermediate" hypertonic osmolarities in the range of 1.3 – 2 OsM (at pH 3.8) (7). Indicators of outer and cytoplasmic membrane changes (crystal violet, SYTO® 9) suggest that *E. coli* is most protected at "intermediate", hypertonic osmolarities (9). From the present study, however, cardiolipin content alone cannot fully explain osmolyte protection of *E. coli* against acetic acid. Nevertheless, the results show that cardiolipin-enrichment of the cytoplasmic membrane occurs in both *E. coli* and *S. enterica* in response not only to osmotic (both hypotonic and hypertonic), but also acid stress, and particularly acetic acid stress.

For both *E. coli* and *S. enterica*, the cardiolipin content (as determined by rsNAO) significantly increased among cell populations with increasing exposure time

under non-growing conditions, even under isotonic conditions. The proportion of *E. coli* cells displaying rsNAO was increased at decreasing pH, achieved by addition of acetic acid, and was also increased in the presence of acetic acid compared with hydrochloric acid at the same pH. Therefore, it was suggested that acetic acid itself imposes a stress that elicits cardiolipin-enrichment. Any advantage to the cell that might be conferred by cardiolipin-enrichment in the presence of acetic acid is not known. No published studies have examined the effect of acetic acid on cardiolipin production in *E. coli* or *Salmonella*, under either growth or no-growth conditions. However, in one study with *E. coli*, at least 90% of phosphatidylglycerol molecules were converted into cardiolipin after incubation with the uncoupling agent CCCP for 90 min (19). It was proposed (15) that in the presence of an uncoupling agent, cardiolipin enrichment assists cell survival by serving as a proton trap, channelling H^+ into the F_1F_0 ATPase, with which it is co-located (14). In this manner, cardiolipin may diminish the pH gradient across the cytoplasmic membrane, while enhancing membrane potential (11). Given that one of the key proposed mechanisms of acetic acid inactivation is collapse of the transmembrane pH gradient (4) and uncoupling (3), it is tempting to speculate that cardiolipin enrichment occurring in the presence of acetic acid may assist cell survival via its ability to modulate proton conductance.

Observations of membrane potential changes made in the present study partially support the hypothesis that cardiolipin-enrichment benefits survival during exposure to acetic acid. Comparing *E. coli* and *S. enterica* cell populations with

and without cardiolipin-enrichment, more cells were depolarised at the end of the 72 h exposure period where cardiolipin-enrichment did not occur. However, at shorter exposure times a smaller proportion of non-cardiolipin-enriched cells were depolarised. Further, for non-cardiolipin-enriched *E. coli* "intermediate" osmolarities appeared to protect cells against depolarisation at shorter exposure times. Taken together, the results for *E. coli* suggest that any advantage of cardiolipin production to cell survival in the presence of acetic acid is complex. At longer exposure times and at both "lower" and "higher" osmolarities, cardiolipin production may benefit cells by maintaining membrane potential. However, at shorter exposure times in the presence of "intermediate" (hypertonic) osmolarities, cardiolipin production may disadvantage the overall survival of the population.

A possible mechanism by which cardiolipin could disadvantage cell survival is related to that by which it may benefit cell survival. Petit et al. (29) showed that there is an unequal distribution of cardiolipin between the inner and outer leaflets of the mitochondrial membrane bilayer (29) that could be mirrored in the bacterial cytoplasmic membrane. By collecting protons (11), cardiolipin may serve as an interfacial reservoir to donate protons to acetate ions in a manner already observed for free fatty acids (2). Thus, the local concentration of undissociated acetic acid at the outer face of the cytoplasmic membrane could increase, as could the diffusion of this species and the rate of uncoupling. However cardiolipin has also been shown to reduce leak conductance and proton conductance by

simple diffusion (2), which might help to explain why cardiolipin production appears to protect *E. coli* populations against depolarisation from exposure to hydrochloric rather than acetic acid.

It is difficult to correlate findings related to cardiolipin-enrichment gathered using single cell flow cytometry studies with those from fluorescence polarisation studies of the bulk population. Nevertheless, beyond the apparent effects of cardiolipin-enrichment on membrane potential, cardiolipin-enrichment may be involved in a general decrease in membrane fluidity. Baronofsky et al. (4) speculated that cells with alterations to membrane lipids might be significantly more impermeable to acetic acid, and Xiang and Anderson (46) showed that deviation of the acetic acid permeability coefficient from that predicted by bulk solubility-diffusion was most marked for densely packed gel-state bilayers. Cardiolipin-enrichment may, thus have implications for the permeability of the undissociated acetic acid species and therefore the rate of cytoplasm acidification. Furthermore, cardiolipin-enrichment could affect the permeability of the ionic species, and thereby other proposed mechanisms of acetic acid inactivation, namely uncoupling (3) and acetate anion accumulation (37).

The reduced inactivation of *E. coli* and *S. enterica* by acetic acid at lower temperatures (28, 43) might also be explained by a decreased rate of diffusion of acetic acid resulting from decreased membrane fluidity (24, 36). There are no reports of the effect of cold on cardiolipin production in *E. coli* or *S. enterica*.

Based on previous observations of membrane changes using SYTO® 9 (9), it was speculated that cardiolipin production might decrease during cold storage. However, this was not the case and rsNAO-fluorescence significantly increased for both *E. coli* and *S. enterica* at 5°C compared with at 23°C. *E. coli* membranes were less fluid at 5°C than at 23°C. Given the enzymatic nature of the production of cardiolipin by cardiolipin synthase (38), it is difficult to understand how cardiolipin production could occur more quickly at 5°C. It is also difficult to reconcile previous observations (9) of low levels of SYTO® 9-staining with rsNAO-staining for cells stored at 5°C. However, a possible explanation lies within the concepts of homeoviscous adaption (41) or the expanded conservation of dynamic membrane properties (12). Given the decrease in membrane fluidity that occurs at lower storage temperatures, cells will work to maintain membrane fluidity, for example by changing the fatty acid composition of their phospholipids (41). On returning cold-adapted cells to a warmer environment, further changes will be required. Given that cells were incubated after their return to room temperature for 1 h in order to permit NAO staining, it is feasible that rapid cardiolipin production may have occurred during this staining period in line with the added pressure to adjust membrane fluidity in response to temperature upshift. Staining with SYTO® 9 in comparison occurred over a shorter (15 min) period that may not have allowed sufficient time for extensive cardiolipin production. Regardless, fluorescence polarisation indicated that cold storage significantly decreased the membrane fluidity of *E. coli* membranes.

In line with the apparent increase in cardiolipin production with increasing exposure time, there was also a general decrease in membrane fluidity (increase in DPH and TMA-DPH polarisation). For both *E. coli* and *S. enterica* a complex bimodal response of membrane fluidity to increasing osmolarity was observed at most sample times. In many cases, at "intermediate" hypertonic osmolarities, an increase in membrane fluidity (assessed as a decrease in DPH and TMA-DPH polarisation) was seen. It is possible that increased membrane fluidity is a response to domain boundary instability (26), which is known to increase with, for example, increasing salinity (45). A possible explanation for the second increase in membrane fluidity observed at "high" osmolarities could be a lamellar to hexagonal phase transition of cardiolipin (12, 27 45), which is induced by both protons and monovalent cations (39). However, additional effects on membrane fluidity beyond those exerted by cardiolipin production are also possible.

In conclusion, time osmolarity, temperature, pH and acidulent all have potential to affect the production of cardiolipin by *E. coli* and *S. enterica*, and cardiolipin content of cell membranes has been shown to coincide with changes in both membrane potential and membrane fluidity. A direct relationship between cardiolipin-enrichment and *E. coli* and *S. enterica* inactivation and survival under non-growing conditions cannot yet be elucidated. However, this study and discussion have shown that cardiolipin-enrichment can affect all of the key proposed mechanisms of acetic acid inactivation postulated to be mediated at the cytoplasmic membrane, namely cytoplasmic acidification, uncoupling and acetate

anion accumulation. This new knowledge of cardiolipin production in response to acetic acid stress complements that of cardiolipin production in response to osmotic stress.

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TABLE 1. Nutrient broth (NB) formulations used in experiments

Formulation designation	Osmolarity (OsM)	NaCl % [a]	sucrose %	Initial expected tonicity [b]
J	0.17	0.5	0	hypotonic
A	0.34	1	0	isotonic
AS	0.34	0.5	6.2	isotonic
B	0.68	2	0	hypertonic
C	1.02	3	0	hypertonic
H	1.21	3	10	hypertonic
D	1.34	4	0	hypertonic
E	1.68	5	0	hypertonic
F	1.99	6	0	hypertonic
G	2.33	7	0	hypertonic

[a] All concentrations are % wt/wt-on-water (i.e. g solute per 100g of water used to prepare broth, and include 0.5% NaCl present in NB base; [b] In minimal medium the growth rate of *E. coli* has been found to be maximal at ~0.3 OsM (external) (32) and this is assumed to represent isotonicity.

TABLE 2. Significance (two-factor analysis of variance) of time and osmolytes (various factors) on *E. coli* cardiolipin production, membrane potential and fluidity

Membrane structure / state	Factor 2	Significance		Average for Formulation / Osmolarity (OsM)										Average at time (h)			
		Time (F1)	F2[c]	J	A	AS	B	C	H	D	E	F	G				
				0.17	0.34	0.34	0.68	1.02	1.21	1.34	1.68	1.99	2.33	6	24	48	72
cardiolipin (rsNAO)[a]	[NaCl]	S[d]	S	67	56	53	63	63	68	69	80	84	90	61	51	83	92
	OsM 1	S	S											61	50	83	91
	OsM 2	S	S											60	50	82	91
	Solute A(S)	S	NS[e]											33	23	76	87
depolarised[a] (rsNAO)	[NaCl]	S	NS	62	53	63	63	63	79	63	65	69	62	35	66	74	74
	OsM 1	S	S											40	67	76	75
	OsM 2	S	NS											41	68	77	76
	Solute A(S)	S	NS											19	51	82	81
depolarised[a] (non-rsNAO)	[NaCl]	S	S	46	60	56	70	62	56	54	52	62	79	36	56	63	88
	OsM 1	S	S											35	55	62	88
	OsM 2	S	S											34	54	62	88
	Solute A(S)	S	NS											49	44	58	81
membrane fluidity (DPH)[b]	[NaCl]	S	S	87	112	100	112	104	115	103	115	115	107	80	111	121	117
	OsM 1	S	S											80	112	121	118
	OsM 2	S	S											80	112	120	114
	Solute A(S)	S	S											74	113	124	113
membrane fluidity (TMA-DPH)[b]	[NaCl]	S	S	79	109	99	114	106	115	103	112	112	104	70	111	122	117
	OsM 1	S	S											70	113	123	118
	OsM 2	S	S											70	111	122	116
	Solute A(S)	S	S											67	113	124	113

[a] Units are %; [b] Units are mP, larger values indicate less fluid; [c] Factor 2 (as per analysis description); [d] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S1)); [e] Not significantly different ($p > 0.05$); For cardiolipin only, underlining indicates response is not significantly different ($p > 0.05$) from response at isotonicity with NaCl (i.e. Formulation A, 0.34 OsM), or response after 24 h exposure, as appropriate.

TABLE 3. Significance (two-factor analysis of variance) of time and osmolytes (various factors) on *S. enterica* cardiolipin production, membrane potential and fluidity

Membrane structure / state	Factor 2	Significance		Average for Formulation / Osmolarity (OsM)										Average at time (h)			
		Time (F1)	F2[c]	J	A	AS	B	C	H	D	E	F	G				
				0.17	0.34	0.34	0.68	1.02	1.21	1.34	1.68	1.99	2.33	6	24	48	72
cardiolipin (rsNAO)[a]	[NaCl]	S[d]	S	86	82	74	87	91	90	92	94	92	93	87	87	92	92
	OsM 1	S	S											88	87	91	92
	OsM 2	S	S											85	87	91	92
	Solute A(S)	S	S											63	74	84	91
depolarised[a] (rsNAO)	[NaCl]	S	S	74	81	77	79	76	73	72	66	68	59	65	79	69	75
	OsM 1	NS[e]	S											66	78	70	74
	OsM 2	NS	NS											67	77	69	73
	Solute A(S)	NS	NS											74	85	79	77
depolarised[a] (non-rsNAO)	[NaCl]	S	S	63	76	67	73	69	73	81	84	88	91	62	81	83	86
	OsM 1	S	S											60	80	83	87
	OsM 2	S	S											59	79	82	86
	Solute A(S)	S	S											62	73	74	76
membrane fluidity (DPH)[b]	[NaCl]	S	S	75	96	97	99	96	107	90	105	106	98	69	104	104	105
	OsM 1	S	S											71	105	105	106
	OsM 2	S	S											72	104	106	106
	Solute A(S)	S	NS											62	98	114	113
membrane fluidity (TMA-DPH)[b]	[NaCl]	S	S	92	109	104	109	110	119	104	113	109	105	85	111	114	117
	OsM 1	S	S											85	112	116	118
	OsM 2	S	S											85	111	116	117
	Solute A(S)	S	NS											62	98	114	113

[a] Units are %; [b] Units are mP, larger values indicate less fluid; [c] Factor 2 (as per analysis description); [d] Interaction between Factor 1 and Factor 2; [d] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S2)); [e] Not significantly different ($p > 0.05$); For cardiolipin only, underlining indicates response is not significantly different ($p > 0.05$) from response at isotonicity with NaCl (i.e. Formulation A, 0.34 OsM), or response after 24 h exposure, as appropriate.

TABLE 4. Significance (single factor analysis of variance) of NaCl concentration and osmolarity on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

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Micro-organism	Membrane structure / state	Analysis abbreviation	Degrees of freedom	Significance at sample time (h)			
				6	24	48	72
E. coli	cardiolipin (rsNAO)	[NaCl]	7	S[a]	S	NS[b]	NS
		OsM 1	8	S	S	NS	NS
	depolarised (rsNAO)	[NaCl]	7	S	S	NS	NS
		OsM 1	8	NS	NS	S	NS
	depolarised (non-rsNAO)	[NaCl]	7	S	S	S	S
		OsM 1	8	S	S	S	S
	membrane fluidity (DPH)	[NaCl]	7	NS	S	S	S
		OsM 1	8	NS	S	S	S
	membrane fluidity (TMA-DPH)	[NaCl]	7	NS	S	S	S
		OsM 1	8	NS	S	S	S
S. enterica	cardiolipin (rsNAO)	[NaCl]	7	S	S	NS	NS
		OsM 1	8	S	S	NS	NS
	depolarised (rsNAO)	[NaCl]	7	NS	S	NS	NS
		OsM 1	8	NS	NS	NS	NS
	depolarised (non-rsNAO)	[NaCl]	7	S	S	S	S
		OsM 1	8	S	S	S	S
	membrane fluidity (DPH)	[NaCl]	7	S	S	S	NS
		OsM 1	8	S	S	S	NS
	membrane fluidity (TMA-DPH)	[NaCl]	7	NS	S	S	S
		OsM 1	8	NS	S	S	S

[a] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S3)); [b] Not significantly different ($p > 0.05$).

TABLE 5. Significance (two-factor analysis of variance) of time and temperature on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

Micro-organism	Membrane structure / state	Significance		Average at temperature (°C)	
		Time (F1)	Temp (F2)	23	5
E. coli	cardiolipin (rsNAO)[a]	S[c]	S	63	70
	depolarised[a] (rsNAO)	S	S	63	78
	depolarised[a] (non-rsNAO)	S	S	62	33
	membrane fluidity (DPH)[b]	S	S	104	129
	membrane fluidity (TMA-DPH)[b]	S	S	106	128
S. enterica	cardiolipin (rsNAO)[a]	NS[d]	S	91	96
	depolarised[a] (rsNAO)	NS	NS	76	72
	depolarised[a] (non-rsNAO)	S	NS	69	73
	membrane fluidity (DPH)[b]	S	NS	96	103
	membrane fluidity (TMA-DPH)[b]	S	NS	110	112

5

[a] Units are %; [b] Units are mP, larger values indicate less fluid; [c] Significantly different ($p \leq 0.05$; *p*-values are provided in the Supplemental material (Table S4)); [d] Not significantly different ($p > 0.05$).

TABLE 6. Significance (two-factor analysis of variance) of time and pH on *E. coli* cardiolipin production, membrane potential and fluidity

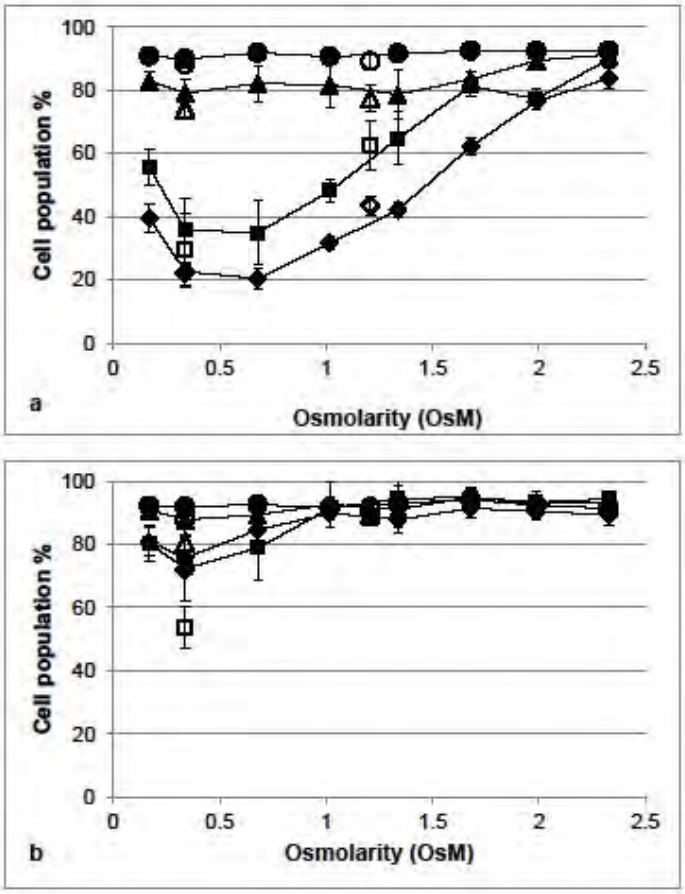
Membrane structure / state	Significance		Average at pH			Significance	
	Time (F1)	pH (F2)	pH 3.6 acetic acid	pH 3.8 acetic acid	pH 4.0 acetic acid	pH 3.6 acetic acid vs. pH 3.8 acetic acid	pH 3.8 acetic acid vs. pH 4.0 acetic acid
cardiolipin (rsNAO)[a]	S	S	64	56	44	NS[d]	S
depolarised[a] (rsNAO)	S	S	70	53	44	S	NS
depolarised[a] (non-rsNAO)	S	S	66	60	55	NS	NS
membrane fluidity (DPH)[b]	S	S	99	112	105	S	NS
membrane fluidity (TMA-DPH)[b]	S	S	100	109	103	S	NS

[a] Units are %; [b] Units are mP, larger values indicate less fluid; [c] Significantly different ($p \leq 0.05$; *p*-values are provided in the Supplemental material (Table S5)); [d] Not significantly different ($p > 0.05$).

TABLE 7. Significance (two-factor analysis of variance) of time and acidulent on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

Micro-organism	Membrane structure / state	Significance			Average with acidulent		
		Time (F1)	Acid (F2)				
			HCl pH 3.6 vs. acetic acid pH 3.6	HCl pH 3.6 vs. acetic acid pH 4.0	HCl pH 3.6	acetic acid pH 3.6	acetic acid pH 4.0
E. coli	cardiolipin (rsNAO)[a]	S[c]	S	na[e]	27	64	na
	depolarised[a] (rsNAO)	S	S	na	32	70	na
	depolarised[a] (non-rsNAO)	S	S	na	50	66	na
	membrane fluidity (DPH)[b]	S	NS[d]	na	96	99	na
	membrane fluidity (TMA-DPH)[b]	S	NS	na	94	100	na
S. enterica	cardiolipin (rsNAO)[a]	S	na	S	89	na	82
	depolarised[a] (rsNAO)	NS	na	NS	82	na	81
	depolarised[a] (non-rsNAO)	S	na	S	58	na	76
	membrane fluidity (DPH)[b]	S	na	NS	94	na	96
	membrane fluidity (TMA-DPH)[b]	S	na	S	102	na	109

[a] Units are %; [b] Units are mP, larger values indicate less fluid; [c] Significantly different ($p \leq 0.05$; p-values are provided in the Supplemental material (Table S6)); [d] Not significantly different ($p > 0.05$); [e] Not applicable



5 FIG. 1. Average percentage of (a) *E. coli* and (b) *S. enterica* cells containing elevated amounts of cardiolipin (i.e. red shifted nonyl acridine orange) after 6 (squares), 24 (diamonds), 48 (triangles) and 72 h (circles) exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard error of the mean (n = 3).

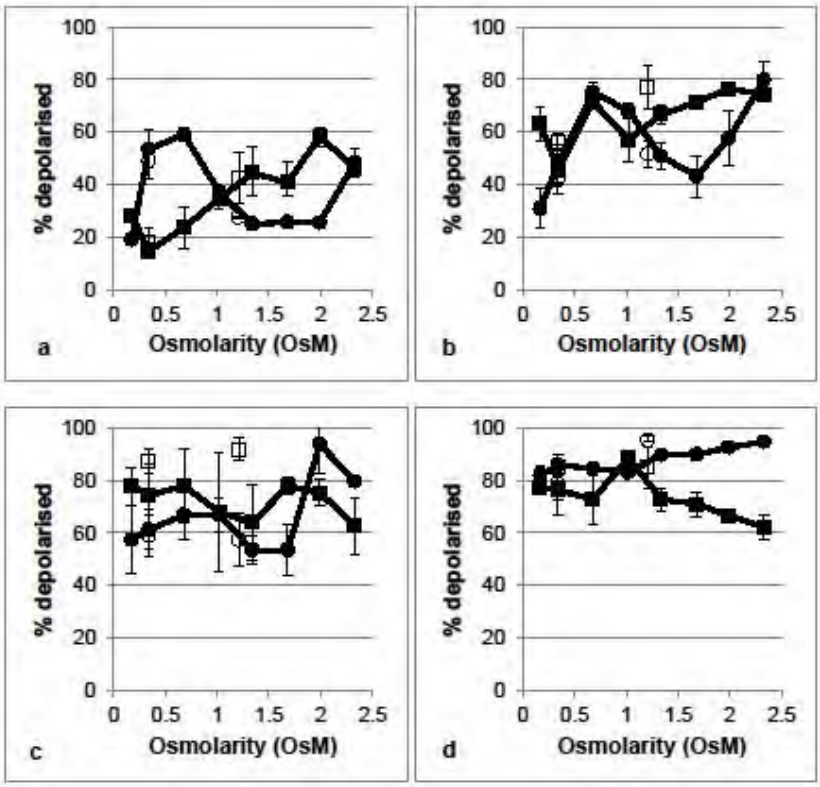


FIG. 2. Average percentage of depolarised *E. coli* cells with (squares) and without (circles) red shifted nonyl acridine orange fluorescence, measured after 6 (a), 24 (b), 48 (c) and 72 h (d) exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard error of the mean (n = 3).

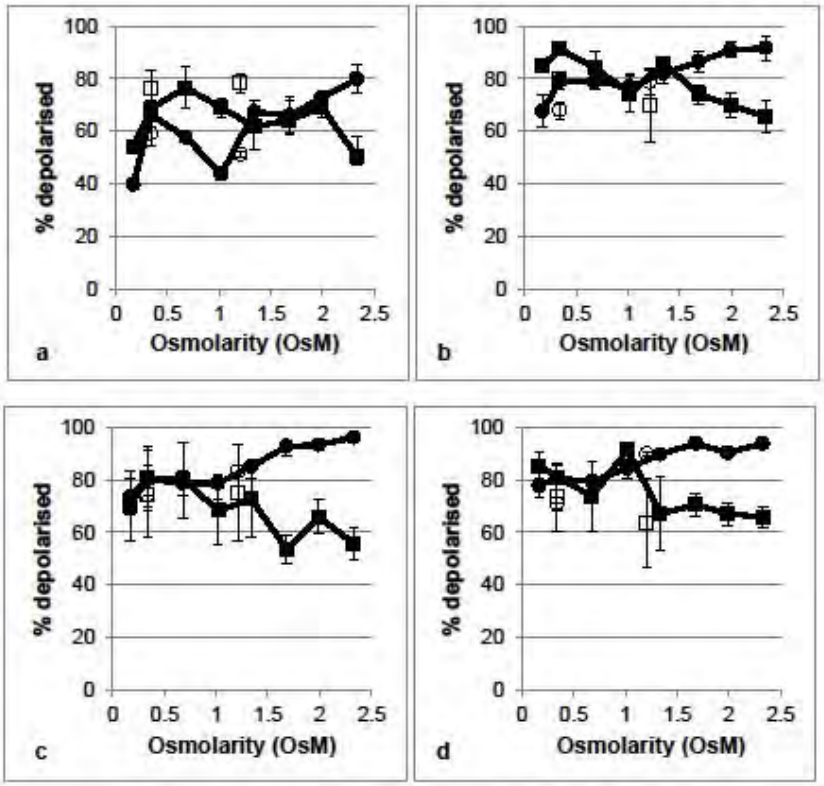
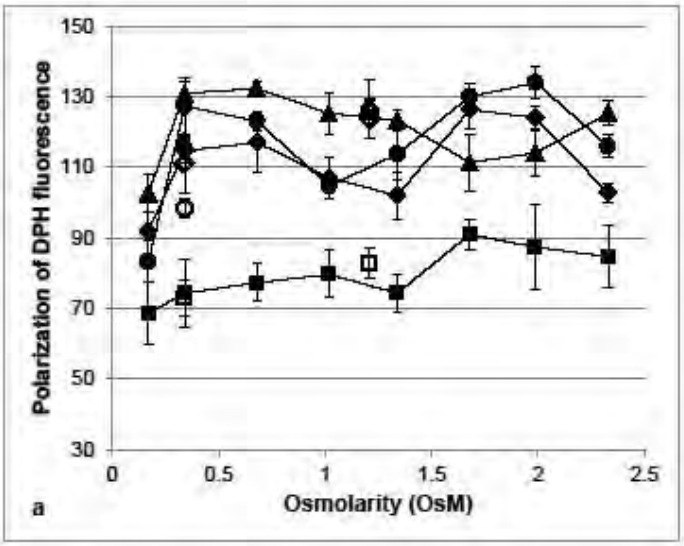


FIG. 3. Average percentage of depolarised *S. enterica* cells with (squares) and without (circles) red shifted nonyl acridine orange fluorescence, measured after 6 (a), 24 (b), 48 (c) and 72 h (d) exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard error of the mean (n = 3).


Polarization of DPH fluorescence
150
130
110
90
70
50
30
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
a

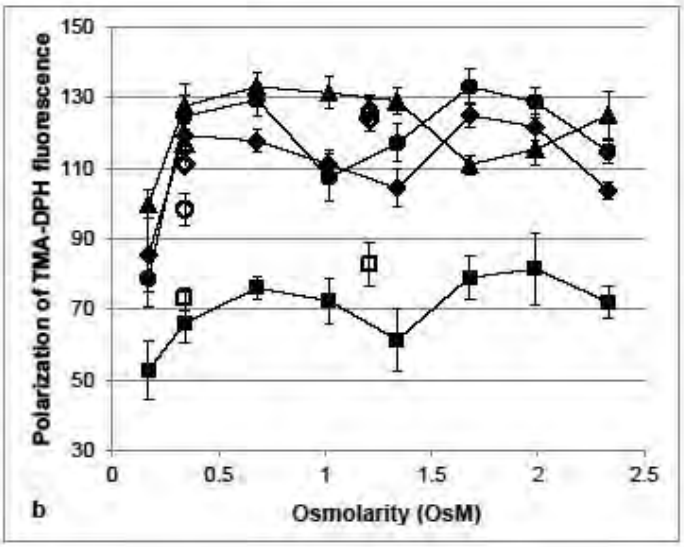
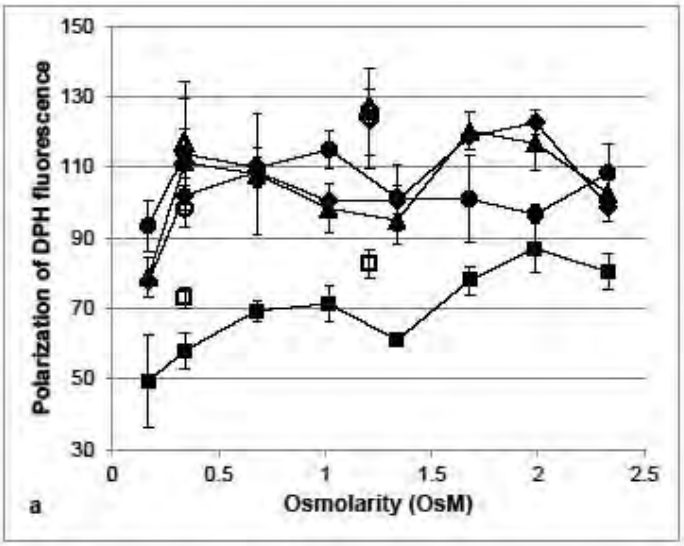

Polarization of TMA-DPH fluorescence
150
130
110
90
70
50
30
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
b

FIG. 4. Average polarisation of (a) DPH and (b) TMA-DPH inserted in *E. coli* cells after 6 (squares), 24 (diamonds), 48 (triangles) and 72 h (circles) exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard error of the mean (n = 3).


Polarization of DPH fluorescence
150
130
110
90
70
50
30
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
a

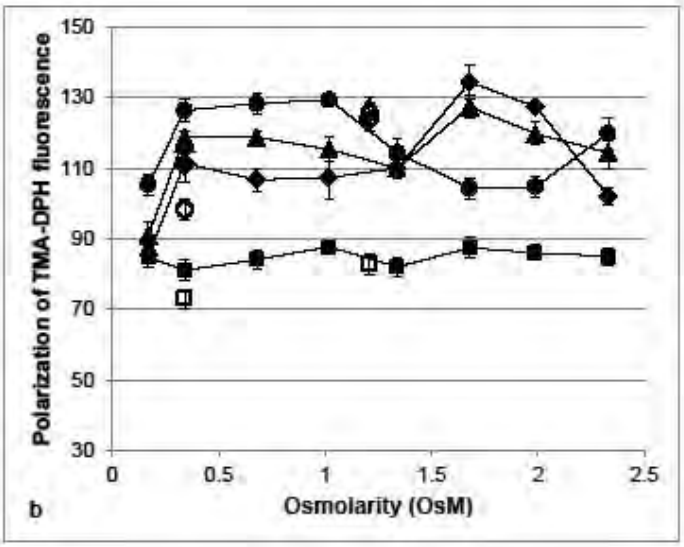

Polarization of TMA-DPH fluorescence
150
130
110
90
70
50
30
0
0.5
1
1.5
2
2.5
Osmolarity (OsM)
b

FIG. 5. Average polarisation of (a) DPH and (b) TMA-DPH inserted in *S. enterica* cells after 6 (squares), 24 (diamonds), 48 (triangles) and 72 h (circles) exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard error of the mean (n = 3).

Supplemental material to -

Cardiolipin production by *Escherichia coli* and *Salmonella enterica* in response to time, osmolytes, temperature and pH during exposure to acetic acid, and changes in membrane potential and fluidity

B. Chapman, T. Ross

Tables S1 – S7.

TABLE S1. Summary of two-factor ANOVA statistical analyses of *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity in response to exposure time, and osmolytes, storage temperature, pH and acidulent type

5

Analysis abbreviation	Analysis description	Treatments (refer to Table 1) and exposure times included in analysis	Degrees of freedom			n[d]
			F1[a]	F2[b]	X[c]	
[NaCl]	Two-factor analysis of time and NaCl concentration	J, A, B, C, D, E, F and G all at pH 3.8 and 23°C at 6, 24, 48, and 72 h	3	7	21	32
OsM 1	Two-factor analysis of time and osmolarity, including sucrose containing formulation H	J, A, B, C, H, D, E, F and G all at pH 3.8 and 23°C at 6, 24, 48, and 72 h	3	8	24	36
OsM 2	Two-factor analysis of time and osmolarity as for OsM1 but replacing formulation A with AS	J, AS, B, C, H, D, E, F and G all at pH 3.8 and 23°C at 6, 24, 48, and 72 h	3	8	24	36
Solute A(S)	Two-factor analysis of time and osmolyte type (NaCl vs. sucrose)	A and AS at pH 3.8 and 23°C at 6, 24, 48, and 72 h	3	1	3	8
Temp C	Two-factor analysis of time and storage temperature	C at pH 3.8, 5°C and 23°C at 24, 48 and 72 h	2	1	2	8
pH A (*E. coli* only)	Two-factor analysis of time and pH	A at pH 3.6, pH 3.8 and pH 4.0 and 23°C at 6, 24, 48 and 72 h	3	2	6	12
Acid A1 (*E. coli* only)	Two-factor analysis of time and acidulent type	A at pH 3.6 with acetic acid & HCl at 23°C at 6, 24, 48 and 72 h	3	1	3	8
Acid A2 (*S. enterica* only)	Two-factor analysis of time and acid / pH	A at pH 4.0 with acetic acid & at pH 3.6 with HCl at 23°C at 6, 24, 48 and 72 h	3	1	3	8

[a] Factor 1 (time) ; [b] Factor 2 (as per analysis description); [c] Interaction between Factor 1 and Factor 2; [d] Number of samples included in analysis

TABLE S2. Significance (two-factor analysis of variance) of time and osmolytes (various factors) on *E. coli* cardiolipin production, membrane potential and fluidity

Membrane structure / state	Factor 2	*p*-value		
		Time (F1)	F2[a]	X[b]
cardiolipin (rsNAO)	[NaCl]	6.0E-21	7.4E-11	6.1E-05
	OsM 1	4.4E-24	2.5E-11	6.5E-05
	OsM 2	9.9E-24	2.6E-12	1.0E-04
	Solute A(S)	2.3E-08	NS[c]	NS
depolarised (rsNAO)	[NaCl]	1.5E-14	NS	NS
	OsM 1	1.8E-13	0.016	NS
	OsM 2	2.6E-14	NS	NS
	Solute A(S)	1.1E-07	NS	NS
depolarised (non-rsNAO)	[NaCl]	1.1E-14	5.9E-10	4.6E-04
	OsM 1	8.4E-29	4.2E-10	3.7E-04
	OsM 2	8.4E-29	2.5E-10	2.9E-04
	Solute A(S)	2.3E-04	NS	NS
membrane fluidity (DPH)	[NaCl]	2.6E-21	3.3E-08	0.011
	OsM 1	1.8E-24	8.4E-09	0.016
	OsM 2	4.2E-24	1.3E-09	0.011
	Solute A(S)	1.9E-06	0.013	NS
membrane fluidity (TMA-DPH)	[NaCl]	8.6E-27	6.1E-11	0.019
	OsM 1	1.2E-31	3.0E-12	0.011
	OsM 2	1.0E-31	3.8E-13	4.8E-03
	Solute A(S)	1.7E-08	8.9E-03	NS

[a] Factor 2 (as per analysis description); [b] Interaction between Factor 1 and Factor 2; [c] Not significantly different ($p>0.05$).

TABLE S3. Significance (two-factor analysis of variance) of time and osmolytes (various factors) on *S. enterica* cardiolipin production, membrane potential and fluidity

Membrane structure / state	Factor 2	*p*-value		
		Time (F1)	F2[a]	X[b]
cardiolipin (rsNAO)	[NaCl]	2.4E-06	6.0E-10	3.0E-04
	OsM 1	2.4E-06	6.9E-10	5.1E-04
	OsM 2	1.6E-07	1.8E-17	6.8E-08
	Solute A(S)	7.7E-06	8.4E-04	NS[c]
depolarised (rsNAO)	[NaCl]	4.7E-03	7.9E-03	NS
	OsM 1	NS	0.041	NS
	OsM 2	NS	NS	NS
	Solute A(S)	NS	NS	NS
depolarised (non-rsNAO)	[NaCl]	6.1E-21	2.0E-17	NS
	OsM 1	2.3E-24	8.8E-17	7.5E-03
	OsM 2	1.7E-23	1.5E-17	0.011
	Solute A(S)	7.2E-03	7.4E-03	NS
membrane fluidity (DPH)	[NaCl]	2.4E-18	8.3E-08	NS
	OsM 1	3.0E-20	1.3E-08	NS
	OsM 2	8.4E-20	9.2E-09	NS
	Solute A(S)	1.6E-04	NS	NS
membrane fluidity (TMA-DPH)	[NaCl]	3.0E-29	7.1E-12	1.7E-11
	OsM 1	7.1E-34	2.5E-15	9.4E-12
	OsM 2	4.6E-34	3.7E-16	3.9E-12
	Solute A(S)	1.6E-04	NS	NS

[a] Factor 2 (as per analysis description); [b] Interaction between Factor 1 and Factor 2; [c] Not significantly different ($p>0.05$).

TABLE S4. Significance (single factor analysis of variance) of NaCl concentration and osmolarity on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

Micro-organism	Membrane structure / state	Analysis abbreviation	Degrees of freedom	*p*-value at sample time (h)			
				6	24	48	72
E. coli	cardiolipin (rsNAO)	[NaCl]	7	7.8E-05	2.1E-10	NS[a]	NS
		OsM 1	8	9.9E-05	5.3E-11	NS	NS
	depolarised (rsNAO)	[NaCl]	7	6.1E-04	3.4E-03	NS	NS
		OsM 1	8	NS	NS	6.1E-03	NS
	depolarised (non-rsNAO)	[NaCl]	7	3.9E-07	2.1E-03	0.035	2.3E-03
		OsM 1	8	1.4E-07	1.8E-03	0.046	3.9E-04
	membrane fluidity (DPH)	[NaCl]	7	NS	3.6E-03	3.1E-03	7.6E-05
		OsM 1	8	NS	2.2E-03	8.4E-03	3.5E-05
	membrane fluidity (TMA-DPH)	[NaCl]	7	NS	1.5E-03	1.7E-03	1.7E-04
		OsM 1	8	NS	3.7E-04	6.5E-04	8.6E-05
S. enterica	cardiolipin (rsNAO)	[NaCl]	7	5.5E-05	1.8E-08	NS	NS
		OsM 1	8	2.3E-05	2.4E-04	NS	NS
	depolarised (rsNAO)	[NaCl]	7	NS	0.018	NS	NS
		OsM 1	8	NS	NS	NS	NS
	depolarised (non-rsNAO)	[NaCl]	7	3.8E-06	0.014	6.6E-04	2.2E-04
		OsM 1	8	2.0E-05	0.017	4.9E-04	8.5E-05
	membrane fluidity (DPH)	[NaCl]	7	0.02	1.3E-06	7.2E-03	NS
		OsM 1	8	8.8E-03	1.8E-07	5.0E-03	NS
	membrane fluidity (TMA-DPH)	[NaCl]	7	NS	4.7E-06	1.3E-04	7.4E-05
		OsM 1	8	NS	2.6E-06	2.1E-05	9.7E-06

[a] Not significantly different ($p>0.05$).

TABLE S5. Significance (two-factor analysis of variance) of time and temperature on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

Micro-organism	Membrane structure / state	*p*-value		
		Time (F1)	Temp (F2)	X[a]
E. coli	cardiolipin (rsNAO)	2.4E-06	0.019	4.3E-06
	depolarised (rsNAO)	6.9E-03	0.042	NS
	depolarised (non-rsNAO)	1.4E-04	6.0E-18	3.4E-03
	membrane fluidity (DPH)	7.2E-03	4.4E-05	0.035
	membrane fluidity (TMA-DPH)	2.9E-04	2.2E-04	0.036
S. enterica	cardiolipin (rsNAO)	NS[b]	5.4E-04	NS
	depolarised (rsNAO)	NS	NS	NS
	depolarised (non-rsNAO)	7.3E-05	NS	NS
	membrane fluidity (DPH)	5.6E-05	NS	NS
	membrane fluidity (TMA-DPH)	9.4E-10	NS	NS

[a] Interaction between Factor 1 and Factor 2; [b] Not significantly different (p>0.05).

TABLE S6. Significance (two-factor analysis of variance) of time and pH on *E. coli* cardiolipin production, membrane potential and fluidity

Membrane structure / state	*p*-value			*p*-value	
	Time (F1)	pH (F2)	X[a]	pH 3.6 acetic acid vs. pH 3.8 acetic acid	pH 3.8 acetic acid vs. pH 4.0 acetic acid
cardiolipin (rsNAO)	4.5E-11	7.2E-04	NS[b]	NS	5.3E-03
depolarised (rsNAO)	3.3E-09	2.5E-04	NS	6.1E-03	NS
depolarised (non-rsNAO)	4.3E-08	0.041	2.2E-03	NS	NS
membrane fluidity (DPH)	5.3E-11	4.8E-03	NS	3.9E-03	NS
membrane fluidity (TMA-DPH)	8.5E-13	0.016	0.023	0.011	NS

[a] Interaction between Factor 1 and Factor 2; [b] Not significantly different (p>0.05).

TABLE S7. Significance (two-factor analysis of variance) of time and acidulent on *E. coli* and *S. enterica* cardiolipin production, membrane potential and fluidity

Micro-organism	Membrane structure / state	*p*-value			
		Time (F1)	Acid (F2)		X[a]
			HCl pH 3.6 vs. acetic acid pH 3.6	HCl pH 3.6 vs. acetic acid pH 4.0	
E. coli	cardiolipin (rsNAO)	8.3E-03	4.2E-05	na[b]	3.2E-03
	depolarised (rsNAO)	2.6E-03	2.9E-05	na	NS
	depolarised (non-rsNAO)	2.1E-04	2.2E-03	na	5.8E-04
	membrane fluidity (DPH)	3.0E-05	NS[c]	na	0.011
	membrane fluidity (TMA-DPH)	4.5E-05	NS	na	NS
S. enterica	cardiolipin (rsNAO)	1.1E-04	na	0.023	NS
	depolarised (rsNAO)	NS	na	NS	NS
	depolarised (non-rsNAO)	0.023	na	2.9E-06	NS
	membrane fluidity (DPH)	1.1E-04	na	NS	NS
	membrane fluidity (TMA-DPH)	7.9E-09	na	6.9E-03	NS

[a] Interaction between Factor 1 and Factor 2; [b] Not applicable; [c] Not significantly different (p>0.05).

8 General discussion

8.1 Discussion

8.1.1 Overview

The simultaneous application of acid (e.g. acetic acid, e.g. in vinegar) and osmolytes (e.g. salts, sugars) is an example of hurdle technology commonly used in the preservation of a range of foods, including cold-filled acid sauces, dressings and mayonnaises (Booth and Kroll, 1989). Implicit in the hurdle approach is the assumption that as each hurdle is made 'higher', microbiological safety and shelf life will be improved (Lee and Kang, 2009). However, under certain conditions, NaCl and sugars appear to protect *Escherichia coli* and *Salmonella enterica* against inimical acetic acid challenge (Casey and Condon, 2002; McKellar *et al.*, 2002; Larson *et al.*, 2003; Chapman *et al.*, 2006: Results chapter 1; Chapman and Ross, 2009: Results chapter 2; Lee and Kang, 2009; Lee *et al.*, 2010).

In comparison with the study by Casey and Condon (2002) that examined the response of *E. coli* under growth permissive conditions, this study examined the response of *E. coli* and *S. enterica* under non-growth permissive (lower pH) conditions that simulate those found in the aqueous phase of cold-filled acidic sauces, dressings and mayonnaises (Chapman *et al.*, 2006: Results chapter 1; Chapman and Ross, 2009: Results chapter 2). The time series data presented in this study complements the study by McKellar *et al.* (2002), that modelled only the probability of growth or survival of *E. coli* O157:H7 at 72 h, and therefore did not provide information regarding the kinetics of inactivation or protection. Like McKellar *et al.* (2002), in this study observations of osmolyte protection of *E. coli* and *S. enterica* were considered in the context of the major industry guideline for cold-filled acid products, namely the CIMSCEE Code (Chapman *et al.*, 2006: Results chapter 1; Chapman *et al.*, 2010: Results chapter 3).

Beyond new data collected in the course of this study a synthesis and analysis of previously published data permitted the development of simple descriptive models (*ln*(inactivation rate) and

ln(rate of 3-$\log_{10}$ inactivation)) to predict the response of *E. coli* and *S. enterica* to a larger number of predictor variables (i.e. pH, acetic acid, NaCl, sugars, other acids, preservatives and storage temperature) (Chapman *et al.*, 2010: Results chapter 3). These variables were determined to be of relevance to the contemporary manufacture of cold-filled acid products via an industry survey of twelve Australian manufacturers, (Chapman *et al.*, 2010: Results chapter 3).

The results of the modelling were used to inform further studies exploring possible mechanism(s) of osmolyte protection against acetic acid inactivation. In these the cell envelope was specifically considered as a target for antagonism in the survival response of *E. coli* and *S. enterica* to simultaneous acetic acid and osmotic challenge. Changes in outer and cytoplasmic membrane integrity, and cytoplasmic membrane composition, fluidity and Δψ (membrane potential) were examined in response to time, osmolytes, temperature and pH in the presence of acetic acid (Chapman *et al.*, in preparation: Results chapter 4; Chapman and Ross, in preparation: Results chapter 5).

8.1.2 Empirical observation of osmolyte protection against acetic acid inactivation, and effects of other factors

Initial experiments to assess the effects of acetic acid, NaCl and sucrose concentrations, and pH, were conducted with an *E. coli* O157 strain, SERL 2, in 81 broth formulations simulating the aqueous phase of cold-fill sauces, dressings and mayonnaises (Chapman *et al.*, 2006: Results chapter 1). The effects of four factors at each of three levels were assessed; acetic acid (0.7, 1.4, and 2.1% [wt/wt]), NaCl (1, 3, and 8% [wt/wt]), sucrose (10, 20, and 30% [wt/wt]), and pH (3.2, 3.5 and 4.0). Broth models were sampled at pre-determined time intervals for up to 120 h, with a maximum of 16 time points. The factorial matrix was selected to parallel that employed by Jenkins *et al.* (2000) in their development of a boundary model for growth of the major spoilage microorganism *Z. bailii* in cold-filled acid products. The boundary model of Jenkins *et al.* (2000) was produced as a contemporary alternative to the CIMSCEE Code, especially in response to consumer demands for milder tasting (i.e. less acidic) cold-filled products.

In these initial, and subsequent, studies *E. coli* inactivation was typically characterised by a biphasic response, with a sharp decline in numbers following an oftentimes prolonged lag phase (Chapman *et al.*, 2006: Results chapter 1). Inactivation thus was modelled using the log logistic equation. When all other factors were held constant, the time to 3-$\log_{10}$ reduction (t_{3D}) of *E. coli* O157 SERL 2 generally increased with increasing pH and with decreasing acetic acid concentrations, as expected from the literature (*see* Section 2.3.2). However the t_{3D} was non-monotonic, i.e. initially decreasing, but then increasing, in response to increasing NaCl concentrations dependent on pH / acid concentration (Chapman *et al.*, 2006: Results chapter 1), consistent with the observations of others (e.g. McKellar *et al.*, 2002). Increasing sucrose concentration also appeared protective under some conditions, and a non-monotonic trend in the relative kill to increasing osmolarity was observed, regardless of whether NaCl or sucrose dominated in the formulation. However multiplication of the lag time prior to inactivation (t_c in the log logistic model) by the rate of inactivation (k_1 in the log logistic model) did not yield a constant product, suggesting some differences in the specific effects of the formulation parameters (Chapman *et al.*, 2006: Results chapter 1).

As a probability-of-survival modelling exercise, the experimental design of McKellar *et al.* (2002) did not permit observation of inactivation kinetics. Nor is the phenomenon of biphasic pathogen inactivation addressed by CIMSCEE (1992). The work of Tuynenberg Muys (1971), on which the CIMSCEE Code is based, assessed inactivation at only three time points (0, 48 and 72 h). From these data log linear inactivation was assumed. It is likely that the sample times chosen by Tuynenberg Muys (1971) were simply insufficient to capture the detail of the biphasic inactivation curve. However it is also possible that the pre-adaptation of pathogens to acidic conditions, achieved using culture in the presence of 1% glucose, resulted in increased acid tolerance compared with the non-adapted cultures used by Tuynenberg Muys (1971). Buchanan and Edelson (1996) also observed relatively long lag times prior to acid inactivation for *E. coli* pre-cultured in 1% glucose, compared with those of non-adapted cultures.

The results of initial experiments with *E. coli* O157:H7 SERL 2 were confirmed and extended in experiments that demonstrated osmolyte protection against acetic acid inactivation to be widely

conserved among different strains of acid resistant *E. coli* and *S. enterica* (Chapman and Ross, 2009: Results chapter 2). The responses of five other strains of acid-resistant (non-pathogenic) *E. coli* strains and four *S. enterica* strains to increasing NaCl concentrations in the presence of acetic and hydrochloric acids were examined. As previously observed for *E. coli* O157 SERL 2, both t_c and k_1 were non-monotonic with respect to increasing osmolarity in the presence of acetic acid; t_c was selected as the primary measure by which to compare inactivation under different conditions, since generally a larger number of data points were collected for this inactivation phase. For *E. coli*, the inflection points in the non-monotonic response of t_c were dependent on pH. Inflection occurred between 2 and 4% at pH 4.0, 4 and 6% at pH 3.8, and 4 and 7% (wt/wt of water) at pH 3.6. These NaCl concentration ranges are approximately equal to 0.7 – 1.3, 1.3 – 2 and 1.3 – 2.3 OsM, respectively. Assuming isotonicity to be around 1% NaCl or 0.3 OsM (Record *et al.*, 1998), the inflection point in the inactivation response was under (initially) hypertonic conditions. Higher NaCl concentrations were more protective under lower pH conditions, where the concentration of undissociated acetic acid would also be greater. In comparison with *E. coli*, *S. enterica* was inactivated much more rapidly. Similar to *E. coli*, a non-monotonic response to increasing NaCl in the t_c was observed for *S. enterica* in the presence of acetic acid under some conditions; at pH 4.0 up to 1 to 4% NaCl was protective, and at pH 3.8 up to 1 to 2% NaCl delayed the onset of inactivation (Chapman and Ross, 2009: Results chapter 2).

The inactivation response of *E. coli* and *S. enterica* was confirmed to be largely independent of osmolyte type (NaCl, sucrose) (Chapman and Ross, 2009: Results chapter 2). Mildly hypertonic conditions of 10% (wt/wt of water) sucrose with 0.5% NaCl (approximately 0.4 OsM), 3% NaCl alone (approximately 1 OsM), and 10% sucrose with 3% NaCl (approximately 1.2 OsM) were all found to be protective against inimical acetic acid concentrations. However, the non-monotonic nature of the inactivation response to increasing osmolarity was found to be dependent on acid type. In comparison with acetic acid, increasing osmolarity in the presence of hydrochloric acid more often resulted in a monotonic decrease in the average lag time prior to inactivation (Chapman and Ross, 2009: Results chapter 2).

Models based on data available in the published literature were developed as a means to generate further evidence of osmolyte protection of *E. coli* and *S. enterica* against acetic acid inactivation (Chapman *et al.*, 2010: Results chapter 3). For a "typical" (median) Australian cold-filled acetic acid-containing dressing or sauce an increase in the ln(inactivation rate) and the ln(rate of 3-$\log_{10}$ inactivation) in response to increasing disaccharide (sucrose) concentration was predicted for *E. coli*, but not *S enterica*. However, it was noted that very little data was available in the published literature regarding the response of *S. enterica* to disaccharides in the presence of acetic acid. In comparison with disaccharides, the concentration of NaCl accounted for much less variability in the models, and was among the four most influential predictor variables for *S. enterica* only. For *S. enterica*, the effect of increasing NaCl concentration was predicted to be protective, but again, the amount of data available in the published literature on which to construct the model was small, and no inactivation data for NaCl concentrations > 3% (wt/wt) was available (Chapman *et al.*, 2010: Results chapter 3).

For *E. coli*, increasing NaCl concentration in the presence of acetic acid was predicted to increase both the ln(inactivation rate) and the ln(rate of 3-$\log_{10}$ inactivation) (Chapman *et al.*, 2010: Results chapter 3). Although this model prediction is apparently in contrast to the central observation of this thesis, it must be remembered that even within the studies of Chapman *et al.* (2006) and Chapman and Ross (2009) (Results chapters 1 and 2), increasing NaCl concentrations did eventually reduce survival of *E. coli* in the presence of acetic acid. The disparity between the observations of protection presented in this thesis and in the work of others (Casey and Condon, 2002; McKellar *et al.*, 2002; Larson *et al.*, 2003; Lee and Kang, 2009; Lee *et al.*, 2010), and the results of the modelling based on previously published (up to mid-2009) data, is most probably a result of the quality of the available published data on which the models were constructed, as discussed in Chapman *et al.* (2010) (Results chapter 3).

Despite the recognised limitations in constructing models based on data from the published literature, the models clearly identified pH and 1/ absolute temperature to be the most influential variables predicting survival in the presence of acetic acid (Chapman *et al.*, 2010: Results chapter 3). Together, these two variables accounted for at least 50% of the variability in the models, for

both *E. coli* and *S. enterica*. As a result, further studies (i.e. Chapman *et al.*, in preparation: Results chapter 4; Chapman and Ross, in preparation: Results chapter 5) included observations of the effects of storage temperature and pH on cell survival, to complement studies of the effect of osmolarity and time.

8.1.3 Comparison with CIMSCEE and relevance to contemporary manufacture of cold-filled products

With the now ubiquitous use of pasteurised egg in commercially produced cold-filled sauces, dressings and mayonnaise, *Salmonella* is no longer considered as great a concern to product safety as it once was (Smittle, 2000). Similarly, it has been concluded that commercially produced sauces, dressings and mayonnaise are not a high-risk vehicle for EHEC / STEC (Erickson *et al.*, 1995). However, the potential for contamination or recontamination of pasteurized egg and egg products with foodborne pathogens has not been entirely eliminated (WHO/FAO, 2002; NSWFA, 2005; USDA, 2006; NSWFA, 2010), and a number of other ingredients including herbs and spices and onion (Pafumi, 1986; WAFMP, 2006 US FDA, 2010; US FDA 2010b; CFIA, 2010) used increasingly in the manufacture of “gourmet” cold-filled acid dressings and sauces (Sloan, 2004; Pszczola, 2008) are also a potential source of pathogens. While GMP and hazard analysis of critical control points (HACCP) is now predominantly used to control food safety hazards (Michels and Koning, 2000), five of twelve Australian manufacturers of cold-filled acid products surveyed in 2006 reported continued use of the CIMSCEE Code as their primary guide to product safety (Chapman *et al.*, 2010: Results chapter 3). Therefore, the performance and limits of the CIMCEE Code for food safety management remain relevant to industry.

Comparison of fitted t_{3D} estimates with CIMSCEE Code safety equation (Equation 2, *see* Section 2.3.1) predictions showed agreement in predicting safety for the majority of formulations (Chapman *et al.,* 2006: Results chapter 1). However, while all formulations with Σs values of <63 had t_{3D} of >72 h, and all formulations with Σs values of >78 had t_{3D} of <72 h, it was found that not all formulations with Σs values between 64 and 78 resulted in t_{3D} of <72 h. Among this subgroup of 22 formulations predicted to be safe by CIMSCEE, the t_{3D} varied between 7 h (Σs = 78) or less

and 125 h (Σs = 68). The unreliability of the CIMSCEE safety equation (Equation 2, *see* Section 2.3.1) for this subgroup of formulations is illustrated by comparing four formulations with t_{3D} of 84, 64, 39, and 7 h, with Σs values of 77, 77, 76, and 76, respectively (*see* Table 1 in Chapman *et al.*, 2006; Results chapter 1).

Further, CIMSCEE safety predictions were "fail dangerous" for 13 of 81 formulations (Chapman *et al.*, 2006: Results chapter 1). The molal undissociated acetic acid versus molal NaCl-plus-sucrose concentration on water was plotted for each of the tested formulation, and formulations were placed into three categories (R1, R2, and R3) (*see* Figure 1 in Chapman *et al.*, 2006: Results chapter 1). For R1, CIMSCEE predictions agreed with experimental results, and all formulations were considered "unsafe" (i.e., fitted t_{3D} were >72 h). For R3, CIMSCEE predictions were also supported by the experimental data but included formulations that were considered "safe" (i.e., >3-$\log_{10}$ reduction in 72 h) and those that were considered "unsafe." In the latter case, all such formulations were found to have a (relatively) high pH (i.e., pH 4.0). Category R2, however, was characterized by frequent disagreements between CIMSCEE safety predictions and the experimental results (Chapman *et al.*, 2006: Results chapter 1).

Within R2, the following three responses were observed: (i) CIMSCEE was found to correctly predict safe formulations in some cases where the pH was relatively low (pH 3.2); (ii) CIMSCEE correctly predicted unsafe formulations where the pH was relatively high (pH 3.5 and pH 4.0); and (iii) CIMSCEE predicted "safe" formulations where the pH was relatively low (pH 3.2 and pH 3.5) that were not supported by the experimental results (Chapman *et al.*, 2006: Results chapter 1). The last group represents the "fail-dangerous" predictions by CIMSCEE. Among these formulations (and others), the observed *E. coli* t_{3D} initially increased and then decreased with increasing osmolarity (NaCl and sucrose). Relative protection increased with exposure time where the protective effect of NaCl predominated. An analysis of cold-filled acid product formulations (Chapman *et al.*, 2010: Results chapter 3), shows that conditions similar to those for which fail-dangerous predictions had previously been demonstrated (Chapman *et al.*, 2006: Results chapter 1) have contemporary relevance. Thirteen of the 57 surveyed commercial

formulations (*see* Table 2 in Chapman *et al.*, 2010: Results chapter 3) fall into category R2 and have a pH ≤ 3.5 (*see* Figure A1 in Appendix A).

Finally, the survey of Australian manufacturers confirmed a contemporary commercial demand to supply cold-filled acid products pre-chilled for incorporation into ready-to-eat salads and similar products (Chapman *et al.*, 2010: Results chapter 3). The CIMSCEE Code does not include temperature as a variable. The CIMSCEE safety equation (*see* Section 2.2.3) was derived from pathogen inactivation studies conducted at 20°C (Tuynenberg Muys, 1971), and thus cannot be relied upon to make predictions of product safety under refrigeration conditions. The development of inactivation models from the published literature demonstrated the importance of temperature to predictions of *E. coli* and *S. enterica* inactivation in the presence of acetic acid (Chapman *et al.*, 2010: Results chapter 3). While the mechanism of protection against acetic acid inactivation by cold temperature is not yet known, there is evidence that protection is concomitant with slowing of the rate of damage to the outer membrane (Chapman *et al.*, in preparation: Results chapter 4 and discussed further in Section 8.1.4).

8.1.4 Exploration of the cell envelope as a target for antagonism in the inactivation response to acetic acid and osmolytes

Eklund (1989) has stated that, in principle, all important subcellular processes or structures should be investigated for their responses to the action of preservatives such as weak acids. Previous hypotheses put forward to account for the observed protection of *E. coli* by osmolytes in the presence of acetic acid have included the possible presence of a sodium-proton antiporter (Casey and Condon, 2002), the induction of glutamate-dependent acid resistance (Richard and Foster, 2007), maintenance of cell volume avoiding concentration of acidic species and intracellular acidification (Kitko *et al.*, 2010), and (contrarily) increased acidification potentially preventing the accumulation of acetate anions (Hosein *et al.*, 2010) (*see* Section 2.3.4).

All of the proposed mechanisms of bacterial inactivation by acetic acid (i.e. intracellular acidification, energy dissipation and uncoupling, and acetate anion accumulation and toxicity) are

ultimately mediated at the level of the cell envelope, and in particular the cytoplasmic membrane (*see* Section 2.4). The most obvious changes brought about during osmotic stress also relate to changes in the structure (e.g. plasmolysis) and composition (e.g. increased proportion of phospholipids with anionic headgroups in the cytoplasmic membrane) of the cell envelope (*see* Section 2.6). Thus it was hypothesised that the non-monotonic nature of inactivation in response to increasing osmolarity in the presence of acetic acid arose from a non-monotonic pattern of damage to, or changes in, the cell envelope. Improved survival at “moderate” (hypertonic) osmolarities could arise as a result of 1) one type of damage increasing both with decreasing and increasing osmolarity, or 2) one type of damage increasing with increasing osmolarity and a second type of damage increasing with decreasing (hypotonic) osmolarity (or, from another perspective, one type of damage increasing with increasing osmolarity and a second type of damage decreasing with increasing osmolarity).

The cell envelope of *E. coli* and *S. enterica* is comprised of three key structures, namely the cytoplasmic membrane, the periplasm including the cell wall, and the outer membrane (*see* Section 2.5). Each of these structures could present both a target for cell damage, and a target for antagonism explaining osmolyte protection against acetic acid. Within the scope of this thesis both the outer and cytoplasmic membranes have been explored. Changes in the periplasm and cell wall have not been explicitly examined, and thus present an area for future studies. A summary of observed changes in the structure and function of the *E. coli* outer and cytoplasmic membranes, as determined by various measures, and in response to various formulation and storage factors, is presented in Table 8.1.

Table 8.1: Summary of *E. coli* cell envelope structure and function changes in response to various formulation and storage factors

Measure	Nominal target in cell envelope	General / notable response to formulation and storage factors					Reference
		Time (increasing)	Osmolarity (increasing)	Cold	pH (decreasing)	HCl (replacing HAc)	
Recovery on plates	Any	Decreases	U-shaped with inflection at "moderate" (hypertonic) osmolarity	Increases	Decreases	Increases	Chapman *et al.* (2009)
Recovery on plates with bile salts	Outer membrane	Decreases	U-shaped with inflection at "moderate" (hypertonic) osmolarity	Increases	Decreases	Increases	Chapman *et al.* (2009)
Incubation with crystal violet and recovery on plates	Outer membrane	Decreases	U-shaped with inflection at "moderate" (hypertonic) osmolarity	Decreases	Decreases	Not investigated	Chapman *et al.* (in preparation)
HI fluorescence	Outer membrane + / - cytoplasmic membrane	Increases	U-shaped with inflection at "moderate" (hypertonic) osmolarity (48 h)[1]	Decreases	Increases	Not investigated	Chapman *et al.* (in preparation)
SYTO® 9 fluorescence	Outer membrane +/- cytoplasmic membrane	Increases	U-shaped with inflection at "moderate" (hypertonic) osmolarity (24 h)	Decreases	Increases	Not investigated	Chapman *et al.* (in preparation)
PI fluorescence	Cytoplasmic membrane	Static	Increases	Decreases	U-shaped with inflection at pH 3.8	Not investigated	Chapman *et al.* (in preparation)

Table 8.1: Summary of *E. coli* cell envelope structure and function changes in response to various formulation and storage factors (continued)

Measure	Nominal target in cell envelope	General / notable response to formulation and storage factors					Reference
		Time (increasing)	Osmolarity (increasing)	Cold	pH (decreasing)	HCl (replacing HAc)	
NAO fluorescence (red-shifted)	Cytoplasmic membrane (cardiolipin)	Increases	U-shaped with inflection ~ isotonic osmolarity (6, 24 h)	Increases	Increases	Decreases	Chapman and Ross (in preparation)
NAO fluorescence (bivariate green:red) – total population	Cytoplasmic membrane (membrane potential)	No clear trend	~ ∩-shaped with inflection at "moderate" (hypertonic) osmolarity	Not investigated	Not investigated	Not investigated	Appendix A
DiOC fluorescence – total population	Cytoplasmic membrane (membrane potential)	No clear trend	~ ∩-shaped with inflection at "moderate" (hypertonic) osmolarity	Not investigated	Not investigated	Not investigated	Appendix A
Membrane fluidity measured by DPH	Cytoplasmic membrane (lipid tail region)	Decreases	Bimodal (~ m – shaped) with second inflection at "moderate" (hypertonic) osmolarity	Decreases	Increases	No difference	Chapman and Ross (in preparation)
Membrane fluidity measured by TMA-DPH	Cytoplasmic membrane (head group region)	Decreases	Bimodal (~ m – shaped) with second inflection at "moderate" (hypertonic) osmolarity	Decreases	Increases	No difference	Chapman and Ross (in preparation)

[1] Not statistically significant

8.1.4.1 Outer membrane

Initial studies to determine the effect on outer membrane permeability of increasing osmolarity under acidic conditions were conducted using plate count methods employing sensitivity to bile salts or crystal violet (Chapman and Ross, 2009: Results chapter 2; Chapman *et al.*, in preparation: Results chapter 4). As a consequence of their size and hydrophobic nature, the influx of bile salts and crystal violet is normally retarded by the presence of an intact outer membrane in wild-type *E. coli* and *S. enterica* (Nikaido, 1979). MacConkey agar contains crystal violet and bile salts as selective agents for the isolation of Gram negative bacteria (Okrend *et al.*, 1990) and its use demonstrates the wild-type resistance of such microorganisms to these agents. Crystal violet and bile salts have been previously employed in the quantitation of sub-lethally injured *E. coli* cells exposed to low temperature stress (Chou and Cheng, 2000), and to low pH (HCl) stress (Buchanan and Edelson, 1996). However outer membrane damage to *E. coli* and *S. enterica* by acetic acid has not previously been reported.

Recovery of *E. coli* (strain 2701) in the presence of bile salts suggested that, like the overall inactivation response, damage to the outer membrane in the presence of acetic, but not hydrochloric acid was non-monotonic in response to increasing osmolarity (Chapman and Ross, 2009: Results chapter 2). At pH 3.6 in the presence of acetic acid, the inflection point in the proportion of injured cells was located between ~0.7 – 1.7 OsM (2 and 5% (wt/wt of water) NaCl), i.e. at “moderate” (initially hypertonic) osmolarities (*see* Figure 2 in Chapman and Ross, 2009: Results chapter 2).

A more extensive study using sensitisation to crystal violet confirmed a significantly non-monotonic extent of *E. coli* (strain 2699) outer membrane damage to increasing osmolarity (Chapman *et al.*, in preparation: Results chapter 4). At pH 3.8 in the presence of acetic acid, the inflection point in the proportion of injured cells was located between ~0.3 – 0.7 OsM (*see* Figure 1b and 1c in Chapman *et al.*, in preparation: Results chapter 4). In comparison, the maximum average relative t_c prior to inactivation for *E. coli* 2699 in the presence of acetic acid at pH 3.8 was at ~ 1.3 OsM (*see* Appendix A, Figure A2b). In comparison with *E. coli* (*see* Figure 1a in

Chapman *et al.*, in preparation: Results chapter 4), *S. enterica* (strain 2742) was more rapidly sensitised to crystal violet on exposure to acetic acid (*see* Appendix A, Figure A3). *S. enterica* was also more rapidly sensitised to crystal violet at high osmolarities at neutral pH (*see* Appendix A, Figure A4) than was *E. coli* (*see* Figure 2a in Chapman *et al.*, in preparation: Results chapter 4). After 2 h exposure to high osmolarity (≥ 1.99 OsM) at neutral pH, significantly fewer *E. coli* cells were sensitised to crystal violet (*see* Figure 2a in Chapman *et al.*, in preparation: Results chapter 4) in comparison with pH 3.8 conditions (*see* Figure 1a in Chapman *et al.*, in preparation: Results chapter 4). Thus outer membrane damage to *E. coli* was shown to occur in response to high osmolarity alone, but was also shown to be potentiated by simultaneous exposure to acidic conditions (Chapman *et al.*, in preparation: Results chapter 4).

Continuing the discussion of the effects of acidification on *E. coli* sensitisation to crystal violet, the effect of pH (achieved using acetic acid) is shown in Figure A5 (Appendix A); sensitivity was increased at all osmolarities at more acidic pH. It is not known whether this increase in damage represents an increase in damage by decreased pH (i.e. $[H^+]$) *per se*, an increase in the total acetic acid concentration, or an increase in the proportion of undissociated acetic acid at the lower pH, since these effects were not independently tested. In Chapman *et al.* (2006) (Results chapter 1), it was noted that the use of KOH to adjust pH had the potential to confound the results of osmolyte protection studies, since K^+ functions as a (temporary) compatible solute (Beales, 2004). Therefore, in subsequent studies (Chapman and Ross, 2009: Results chapter 2; Chapman *et al.*, in preparation: Results chapter 4; Chapman and Ross, in preparation: Results chapter 5) acetic acid alone (or hydrochloric acid alone, as appropriate) was used to adjust formulations to a given pH. Alakomi *et al.* (2000) described outer membrane damage to *E. coli* by HCl. Buchanan and Edelston (1996) also observed outer membrane damage reducing recovery of *E. coli* on bile salt-containing MacConkey agar, compared with non-selective Brain Heart Infusion agar (BHIA) following exposure to pH 2.5 and 3 adjusted using HCl.

Further study of outer membrane permeability of the total (i.e. not only the recoverable) population used flow cytometry and the fluorescent indicator hexidium iodide (HI) (Chapman *et al.*, in preparation: Results chapter 4). HI has been used as an alternative Gram stain for the

differentiation of Gram positive and Gram negative bacteria, and provides an indication of outer membrane permeability in Gram negative bacteria (Mason *et al.*, 1998). As previously observed for sensitivity to bile salts (Chapman and Ross, 2009: Results chapter 2) and crystal violet (Chapman *et al.*, in preparation: Results chapter 4), permeability to hexidium iodide initially increased more rapidly at high osmolarity (*see* Figure 4a in Chapman *et al.*, in preparation: Results chapter 4). However, as for bile salts and crystal violet, increased permeability of *E. coli* to HI also occurred with increasing exposure time at low (hypotonic, isotonic) osmolarity (*see* Figure 4a in Chapman *et al.*, in preparation: Results chapter 4). While a non-monotonic trend for HI staining of *E. coli* was observed after 48h exposure with an apparent inflection point around 1.5 OsM, this was not statistically significant (Chapman *et al.*, in preparation: Results chapter 4). However, for *S. enterica* a statistically significant non-monotonic response to increasing NaCl concentration, with a minimum around 0.7 OsM was observed (Figure A6, Appendix A). The response to HI staining for both *E. coli* and *S. enterica* (*see* Figure 4a in Chapman *et al.*, in preparation: Results chapter 4, and Figure A6 in Appendix A) appeared more dependent on solute type than did the response of crystal violet sensitisation (*see* Figure 1 in Chapman *et al.*, in preparation: Results chapter 4 and Figure A3 in Appendix A).

With regards the effect of pH, significantly less HI staining was observed at pH 3.8 compared with pH 3.6, but HI-staining of *E. coli* at pH 4.0 was not significantly different from staining at pH 3.8 (Chapman *et al.*, in preparation: Results chapter 4). In comparison with observations of crystal violet sensitivity, permeability to HI suggested less damage in the presence of high NaCl concentrations at neutral pH, with only around 5% of *E. coli* cells showing fluorescence > 38 units (*see* Figure 2b in Chapman *et al.*, in preparation: Results chapter 4).

By all measures (i.e. bile salts, crystal violet and HI) outer membrane permeability increased strongly with increasing exposure time for both *E. coli* and *S. enterica* in response to acid conditions, with outer membrane damage appearing much more rapidly for the latter than the former. Storage temperature also had a significant effect on both crystal violet and HI permeability of *E. coli* cells, with cells incubated at 23°C taking up significantly more stain, inferring greater

outer membrane permeability, than those stored at 5°C (Chapman *et al.*, in preparation: Results chapter 4).

Taken together the results for crystal violet sensitisation and HI-staining suggest that while both assays detect changes in outer membrane permeability, the exact changes inferred may be different. Alternatively, or in addition, changes in HI staining may be confounded by other cell changes affecting HI uptake and / or accumulation (Chapman *et al.*, in preparation: Results chapter 4). For example, the results of Three Dimensional Structured Illumination Microscopy (3D-SIM) suggest that HI-staining may be complicated by an unexpected accumulation in specific cytoplasmic membrane domains (Chapman *et al.*, 2010: Results chapter 3). This is further in Section 8.1.4.2 with reference to SYTO® 9-staining of the cytoplasmic membrane.

While generally referred to as a "membrane permeant" indicator (Stocks, 2004), it has previously been suggested that an increase in SYTO® 9 permeability might indicate outer membrane damage for Gram negative bacteria (Berney *et al.*, 2007). In the present study, it was noted that SYTO® 9-fluorescence strongly increased with increasing exposure times and decreasing pH, and changed non-monotonically with increasing osmolarity (Chapman *et al.*, in preparation: Results chapter 4), as previously observed for other measures of outer membrane permeability (i.e. bile salts, crystal violet, HI). After 24h exposure in the presence of acetic acid at pH 3.8, minimum SYTO® 9-fluorescence was observed around 1 OsM for *E. coli* (*see* Figure 6a in Chapman *et al.*, in preparation: Results chapter 4). For *S. enterica*, an unexpected but statistically significant decrease in the proportion of cells exhibiting SYTO® 9-staining occurred with increasing osmolarity after 2 h exposure at pH 4.0 (Figure A7, Appendix A, Supplemental data). This trend, which disagrees with those observed for crystal violet sensitivity and HI-staining does not yet have a satisfactory explanation. Otherwise, SYTO® 9-fluorescence for *S. enterica* increased with increasing exposure time, in a similar fashion to that observed for *E. coli*. Thus, overall it remains plausible that SYTO® 9-staining is at least partially indicative of changes in outer membrane permeability, as proposed by Berney *et al.* (2007).

8.1.4.2 Cytoplasmic membrane

Outer membrane damage to Gram negative microorganisms has been shown both to arise from, and to potentiate damage to, the cytoplasmic membrane (Bayer, 1968; Nossal and Hepel, 1996; Vásquez-Laslop *et al.*, 2001; Milillo *et al.*, 2011). Therefore, gross damage to the cytoplasmic membrane was assessed using flow cytometry and propidium iodide (PI) (Chapman *et al.*, in preparation: Results chapter 4), which will not enter cells unless the cytoplasmic membrane is compromised (Stocks, 2004). Substantial loss of cytoplasmic membrane integrity as assessed by PI did not occur for any treatment (Chapman *et al.*, in preparation: Results chapter 4). Even after 120 h exposure to high osmolarities ≥ 1.68 OsM in the presence of acetic acid, less than 8% of *E. coli* and 5% of *S. enterica* cells, on average, were discriminated by PI staining (*see* Figure 5 in Chapman *et al.*, in preparation: Results chapter 4, and Figure A8 in Appendix A).

Beyond flow cytometric assays, visualisation of HI and especially SYTO® 9-staining by 3D-SIM (*see* Figure 7 in Chapman *et al.*, in preparation: Results chapter 4) revealed the presence of very brightly stained discrete domains apparently located in the plane of the cytoplasmic membrane. These observations by 3D-SIM suggested an additional interpretation for high intensity SYTO® 9- and HI -fluorescence. Given the cationic nature of SYTO® 9 and HI that facilitates their interaction with anionic nuclear material (Haugland, 1996), we hypothesised that the brightly stained cytoplasmic membrane domains may be rich in the strongly anionic (Lewis and McElhaney, 2009) phospholipid cardiolipin. These membrane domains are initially localised to the polar regions of the cell, but appeared to multiply and distribute throughout the cell with time (Chapman *et al.*, in preparation: Results chapter 4). Using a computational modelling approach, Huang *et al.* (2006) predicted that the number of clusters of cardiolipin in the cylindrical region of a bacterial cell will remain low until cardiolipin concentrations increase to the point where the poles are densely packed with clusters. Subsequent additions of cardiolipin are then predicted to produce clusters that distribute uniformly throughout the cylindrical region of a rod-shaped cell (Huang *et al.*, 2006). Confirmation that the very bright SYTO® 9- and HI -stained cytoplasmic membrane domains contained cardiolipin was undertaken using 3D-SIM and the fluorescent indicator 10-*N*-nonyl

acridine orange (NAO), which has high affinity for cardiolipin (Mileykovskaya and Dowhan, 2000) (*see* Figure 8 in Chapman *et al.*, in preparation: Results chapter 4).

Cardiolipin is one of the three key phospholipids found in the cytoplasmic membrane of *E. coli* (Cronan, 2003; Sections 2.5.3 and 2.6.6), and has been shown to be substantially involved in the response of this microorganism (and others) to osmotic stress (Romanstov *et al.,* 2008; Romantsov *et al.*, 2009). Although cardiolipin-enrichment is not typically considered in the context of acid stress, it has been shown to assist cell survival in the presence of synthetic uncouplers (Lacombe and Lubochinsky, 1988). Thus it was further hypothesised that cardiolipin-enrichment might play a part in the non-monotonic inactivation response of *E. coli* and *S. enterica* to osmolarity in the presence of acetic acid.

Using flow cytometry, the proportion of *E. coli* cells exhibiting red-shifted NAO fluorescence characteristic of interaction with cardiolipin was determined to increase with increasing exposure time and decreasing pH, and to respond non-monotonically to increasing osmolarity (*see* Figure 1a in Chapman and Ross, in preparation: Results chapter 5). However, it was noted that the inflection point of minimum cardiolipin-enrichment was typically at a lower osmolarity (approaching isotonicity) than those previously observed for the average t_c, as well as for other measures of population change, including the proportion of cells exhibiting increased SYTO® 9-fluorescence. It was also observed for *E. coli* that cardiolipin-enrichment was statistically significantly favoured by the presence of acetic acid, compared with hydrochloric acid (*see* Table 8 in Chapman and Ross, in preparation: Results chapter 5). Cardiolipin-enrichment in *S. enterica* occurred very quickly in the presence of both acetic and hydrochloric acids (*see* Figure 1b and Table 8 Chapman and Ross, in preparation: Results chapter 5). Unlike SYTO® 9- staining NAO staining of *E. coli* was not inhibited by storage at 5°C. This observation both suggests that cardiolipin-enrichment proceeds under conditions of cold storage, and confirms that increases in SYTO® 9- fluorescence are not only dependent on cardiolipin-enrichment, but also indicate other cell envelope changes that take place at 23°C but not 5°C.

Nevertheless, the results of this study strongly suggest involvement of cardiolipin in the response of *E. coli* and *S. enterica* to acetic acid stress presented (Chapman and Ross, in preparation: Results chapter 5). *Listeria monocytogenes* grown in the presence of acetic acid at pH 5.5 has been shown to increase cardiolipin content by 13.8% (Mastronicolis *et al.*, 2010). However, cardiolipin involvement in the stress response of *E. coli* and *S. enterica* to acetic acid stress in particular, and acid stress in general, has not previously been reported. There are at least three broad mechanisms by which changes in cardiolipin content could be hypothesised to affect the survival of *E. coli* and *S. enterica* in the presence of acetic acid. Each mechanisms also presents as a potential target for antagonism with the response to osmotic stress. These three broad mechanisms are due to i) changes in membrane permeability, ii) changes in Δψ, and iii) improved efficiency of membrane protein function. During this thesis study specific work was undertaken to address the first two of these proposed mechanisms. The results of those studies are discussed below in the context of the existing literature.

Acidification of the cytoplasm must be dependent both on the rate of acetic acid and proton ingress, typically understood to be by passive diffusion across the cytoplasmic membrane, and the rate of proton egress (e.g. by pumping) (Russell, 1992). If changes in osmolarity affected the rate of proton egress (or ingress), then any observed protection would reasonably be expected to occur in the presence of both hydrochloric and acetic acid. However, since osmolyte protection was observed only for acetic acid (Chapman and Ross, 2009: Results chapter 5), this suggests an effect predominantly on the rate of undissociated acid ingress, which would also be expected to affect the rate of acetate anion accumulation. While acetic acid is generally considered to be lipophilic, it is only weakly so, since the polar acid functional group dominates over the short nonpolar hydrocarbon chain (Steiner and Sauer, 2001; *see* Section 2.5.4). The ability of acetic acid to diffuse through bilayer membranes is known to be susceptible to changes in membrane packing (Xiang and Anderson, 1997), which could be brought about through changes in membrane composition, e.g., cardiolipin enrichment.

Due to its relatively small headgroup size, it has been suggested that an increase in cardiolipin will tend to increase membrane chain ordering, thus forming tightly packed domains with reduced

membrane fluidity (Arcisio-Miranda *et al.*, 2009). However, the onset of domain boundary instabilities between, for example, areas rich and poor in cardiolipin may actually increase cytoplasmic membrane fluidity overall (Epand and Epand, 2009). For example, fluorescence polarisation studies of *Pseudomonas putida* DOT-T1E and its isogenic *cls*$^{-}$ (cardiolipin synthase knock-out) mutant suggested that the mutant membrane, with less cardiolipin than the wild-type membrane, was less fluid (Bernal *et al.*, 2007).

General membrane fluidity was assessed using fluorescence polarisation of the probes DPH and TMA-DPH (Chapman and Ross, in preparation: Results chapter 5), which target the acyl chain and headgroup regions, respectively (Kuhry, 1989). The results of the fluorescence polarisation study indicated a complex, kinetically shifting bimodal response of membrane fluidity to increasing osmolarity in the presence of acetic acid (Chapman and Ross, in preparation: Results chapter 5). For both *E. coli* and *S. enterica*, fluidity appeared greatest (polarisation was minimised) under “low” and “high” osmolarity conditions, but also at some “moderate” osmolarities (*see* Figures 4 and 5 in Chapman and Ross, in preparation: Results chapter 5). Notably, the second inflection point (i.e. the point between the individual distributions) typically occurred at a higher osmolarity than the inflection point for cardiolipin-enrichment already described. There was a clear overall decrease in membrane fluidity after 24 h, at all osmolarities, and as measured by both DPH and TMA-DPH. For *E. coli*, membrane fluidity then appeared to continue to decrease with increasing exposure time, but polarisation values for the first of the bimodal populations began to decrease before the end of the 72 h exposure period (*see* Figure 4 in Chapman and Ross, in preparation: Results chapter 5). In contrast, for *S. enterica* polarisation values for the second of the bimodal populations began to decrease before the end of the 72 h exposure period (*see* Figure 5 in Chapman and Ross, in preparation: Results chapter 5). This complex pattern of changes in membrane fluidity could result from a number of physicochemical changes relatable to cardiolipin-enrichment, including, as summarised by Arcisio-Miranda *et al.*, (2009); an increase in the bound water at the lipid-water interface, an increase in the packing of the headgroup region, an increase in the packing order of acyl chains, a decrease in the adhesive forces between the inner and outer membrane leaflets, and the possible formation of inverted non-lamellar phases that are encouraged by, for example, protonation at low pH.

Beyond the general concept of membrane fluidity, more specific exploration of the effect of cardiolipin content on proton flux, including that induced by the presence of long chain free fatty acids has been undertaken recently (Arcisio-Miranda *et al.*, 2009). The presence of cardiolipin in black lipid membranes of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was found to reduce membrane conductance due to both ion-unspecific leakage, and simple diffusion of H^+ across the bilayer. The authors postulated these reductions in membrane conductance to be as a result of increased ordering of the bilayer, reducing permeability to water, H^+ and other ions. However, in membranes supplemented with oleic acid Arcisio-Miranda *et al.* (2009) found the presence of cardiolipin to substantially increase simple diffusion of H^+, and hypothesised that this increase could be linked to cardiolipin's ability to act as a proton trap (Haines and Dencher, 2002), or antenna (Hoch, 1998), harvesting protons and donating them to the free fatty acid, which then translocated the protons via flip-flop diffusion.

As an extension to their recent work modelling the effects of NaCl and acetic acid on the intracellular pH of *E. coli*, Hosein *et al.* (2011) speculated that future work focused on the proton motive force (ΔP) might help elucidate the relationship between intracellular pH and acetate anion concentration, and cell survival. The presence of cardiolipin is a defining feature of energy producing membranes, since cardiolipin occurs primarily in membranes that also contain the F_oF_1-ATPase (Haines, 2009). Considering the structure of the cardiolipin molecule, a unique role for cardiolipin as a headgroup proton trap has been proposed (Haines and Dencher, 2002). In addition to aggregating the oxidative phosphorylation proteins into a specific membrane domain, cardiolipin may restrict pumped protons within its headgroup domain, directly re-supplying H^+ to the ATP synthase (Haines and Dencher, 2002). In this manner, interventions (such as synthetic uncouplers) that normally diminish the ΔpH would be less effective in diminishing ATP synthesis (Haines and Dencher, 2002). Since Δψ is assumed to be the driving force for ATP synthesis, cardiolipin-enrichment could be expected to sustain Δψ through the increase in charged groups created by its synthesis, even in the absence of ΔpH (Haines and Dencher, 2002).

Although often referred to as a “cardiolipin-specific” stain, NAO accumulation has been shown to be affected by Δψ changes (Keij *et al.*, 2000; Jacobson *et al.*, 2002). Observations of Δψ changes made as part of the NAO staining study suggested that any influence of cardiolipin on Δψ (like that of membrane fluidity) and cell survival in the presence of acetic acid is complex (Chapman and Ross, in preparation: Results chapter 5). Given the potential for confounding of the Δψ results of NAO-staining by the stain’s high affinity for cardiolipin, studies were also undertaken using a second Δψ-sensing dye, $DiOC_2(3)$ (Shapiro, 2000) with *E. coli*. $DiOC_2(3)$ has no particular documented affinity for cardiolipin, and observation by 3D-SIM failed to reveal a pattern of bright cytoplasmic domain staining as previously observed for HI and SYTO® 9 (data not shown). Unfortunately, it has been noted that $DiOC_2(3)$ has poor penetration through the outer membrane of Gram negative bacteria (Shapiro, 2000). If it was assumed that cells maintaining outer membrane function also maintained Δψ, estimates of depolarisation made using NAO for the whole cell population were broadly confirmed by staining with $DiOC_2(3)$ (Figure A9, Appendix A). However more work is required to accurately determine the relationship between cardiolipin enrichment and Δψ.

Considering NAO staining and comparing *E. coli* and *S. enterica* cell populations with and without cardiolipin-enrichment, generally more cells were depolarised at the end of the 72 h exposure period where cardiolipin-enrichment did not occur (Chapman and Ross, in preparation: Results chapter 5). However, at the shortest exposure time of 6h more cardiolipin-enriched than non-cardiolipin-enriched cells were depolarised at “moderate” (initially hypertonic) osmolarities, while more non-cardiolipin-enriched than cardiolipin-enriched cells were depolarised at lower osmolarities around (initial) isotonicity. Therefore, at shorter exposure times in the presence of “moderate” osmolarities, cardiolipin production may disadvantage cell survival. Yet at longer exposure times and at both “lower” and “higher” osmolarities, it is possible that cardiolipin production may benefit cells by maintaining Δψ (Chapman and Ross, in preparation: Results chapter 5). The complexity of the Δψ response may arise from the complexity of the (sometimes competing) actions of cardiolipin as a proton trap for oxidative phosphorylation and a mediator of changes in membrane permeability to H^+ and other ions, as already discussed. However, as for

membrane fluidity, it is also possible that factors other than cardiolipin content contribute to the complexity of the observed Δψ response.

No specific studies of membrane channel efficiency were undertaken during the course of this thesis. As for study of the periplasm and cell wall, this remains an area for future work. A number of osmoregulatory proteins have been shown to be preferentially co-located with cardiolipin, including the mechanosensitive channel MscS, that mediates solute efflux in response to decreasing osmotic pressure (Romantsov *et al.*, 2010) and the H^+-osmolyte symporters ProP, responsible for the inward transport of organic compatible solutes (Tsatskis et al 2005, Romantsov et al 2008). Considering that cardiolipin also aggregates proteins involved in oxidative phosphorylation, it seems reasonable to propose there may be a fine balance in benefits and detriment of cardiolipin-enrichment to functioning of membrane proteins involved in the responses to osmotic and acid stress.

8.2 Conclusion

In conclusion it is appropriate to reflect on the overall aims of this study. The aims of this thesis were; 1) to expand understanding of the protective effects of osmolytes on the inactivation of *E. coli* and *Salmonella enterica* by acetic acid under non-growth permissive conditions; 2) to review the relevance of observations of protection to international industry guidelines and contemporary manufacture of acetic acid containing cold-filled sauces, dressings, and mayonnaises; and 3) to investigate possible mechanisms of protection of osmolytes against inactivation by acetic acid, involving changes in the cell envelope.

An improved understanding of the protective effects of osmolytes under non-growth permissive conditions in the presence of acetic acid has been achieved. Moderately hypertonic concentrations of NaCl and sucrose have been shown to protect *E. coli* and *S. enterica* against inactivation by acetic acid, although not by hydrochloric acid. This protective phenomenon has been shown to be largely independent of osmolyte type, although some minor differences in the response to individual osmolytes (NaCl and sucrose) were noted. Inactivation under the tested

conditions was often biphasic, with protection manifested in both an increase in the lag time prior to inactivation, and a decrease in the inactivation rate following lag. In general, inactivation occurred most quickly at high osmolarities, then at low (hypotonic, isotonic) osmolarities, and least quickly at moderate (hypertonic) osmolarities. Protection was demonstrated to be well conserved among acid resistant strains of *E. coli* and *S. enterica*, although *S. enterica* is much more sensitive to inactivation by acetic acid overall.

The observations of protection made in this study remain of relevance to the contemporary manufacture of acetic acid- containing cold-filled sauces, dressings and mayonnaises. Conditions under which osmolyte protection of *E. coli* and *S. enterica* were observed were confirmed to fall within the formulation recommendations of the CIMSCEE Code (1992), an international industry guideline for the manufacture of microbiologically-safe cold-filled products that remains in current use by industry. A survey of Australian manufacturers of cold-filled acid products confirmed that contemporary formulations included conditions for which osmolyte protection against acetic acid might be expected.

Since the antibacterial activity of acetic acid is usually explained by mechanisms mediated at the level of the cell envelope, and since the most obvious changes in response to osmotic stress also affect the cell envelope, it was hypothesised that the non-monotonic inactivation response might be reflected in non-monotonic changes in cell envelope (outer membrane, cytoplasmic membrane) damage. Specifically, it was proposed that improved survival at moderate (hypertonic) osmolarities could arise from: 1) one type of damage increasing with osmolarity above or below the optima, or 2) one type of damage increasing with increasing osmolarity and a second type of damage increasing with decreasing (hypotonic) osmolarity. In fact, it was shown that at least two of the cell envelope changes that accompany exposure to combined acetic acid and osmotic stress are non-monotonic in nature. Changes in both outer membrane integrity and cardiolipin-enrichment of the cytoplasmic membrane qualitatively correlated with the non-monotonic inactivation pattern of *E. coli* and *S. enterica* in response to combined acetic acid and osmotic stress.

Damage to the outer membrane of *E. coli* and *S. enterica* has not previously been reported for acetic acid, and thus represents a novel insight into the effect of this antibacterial agent. Outer membrane damage was clearly potentiated by decreased pH, but also occurred at neutral pH at high osmolarity. As observed for the overall inactivation response, in the presence of acetic acid outer membrane changes occurred soonest at high osmolarities, followed by low, then moderate osmolarities. Outer membrane damage occurred more quickly for *S. enterica* than for *E. coli*, reflecting the relative sensitivities of these two microorganisms to inactivation by acetic acid. Further, it was clearly demonstrated that the rate of development of outer membrane damage under inimical acid conditions is reduced under conditions of cold storage, which might help to explain the ability of pathogens to persist at low temperatures. Specifically how reducing outer membrane damage might prolong cell survival is not known. While it has been shown by others that outer membrane damage can potentiate damage to the cytoplasmic membrane, this did not appear to be the case for acetic acid damage (Chapman *et al.*, in preparation: Results chapter 4). While the outer membrane is not normally considered as a barrier to small molecules, it is conceivable that changes in the cell-solution interface or outer membrane permeability could affect the rate of ingress of undissociated acid molecules into the periplasmic space, and therefore cell permeability overall.

Cardiolipin-enrichment of the cytoplasmic membrane is well known to occur in response to osmotic stress, but has not previously been reported for *E. coli* and *S. enterica* in response to acetic acid stress. Given the potential for cardiolipin-enrichment to alter membrane permeability, $\Delta\psi$ and the aggregation and therefore efficiency of membrane proteins, it is clear that such change in cytoplasmic membrane composition could affect many elements of cell physiology. In turn, this may result in a complex pattern of benefit and detriment for cell survival, depending on a fine balance of environmental conditions, especially osmolarity and acidity, and exposure time. However, it is also recognised that many other changes in outer and cytoplasmic membrane composition and structure (e.g. sustained plasmolysis) could contribute to the complex changes in membrane fluidity observed in this study. Similarly, $\Delta\psi$ is likely to be affected by other changes in cytoplasmic membrane physiology beyond cardiolipin content.

Although more work remains to correlate the various observed responses it is concluded that, under certain environmental conditions, *E. coli* and *S. enterica* are protected against inactivation by acetic acid by osmolytes. This protection appears concomitant with maintenance of outer membrane integrity and with some increase in the cardiolipin content of the cytoplasmic membrane. Thus it remains a compelling hypothesis that interactions mediated at the cell envelope contribute to osmolyte protection of *E. coli* and *S. enterica* against acetic acid inactivation. A number of further hypotheses and methods for testing antagonism in the response to combined acetic acid and osmotic stress are outlined in Section 8.3, below.

8.3 Further hypotheses for testing

8.3.1 Changes in the cytoplasmic membrane change acetic acid permeability

Changes in membrane composition and structure could affect any or all of undissociated acid, proton or anion permeability (Russell *et al*., 1995; Arcisio-Miranda *et al*., 2009; Section 2.5.4). Based on kinetic observations of sublethal injury in Chapman and Ross (2009) (Results chapter 2), it was hypothesised that the non-monotonic inactivation response to increasing osmolarity in the presence of acetic acid could arise from a decrease in acetic acid permeability with cytoplasmic membrane changes at moderate (hypertonic) osmolarities, coupled with a loss of cell envelope structure and function at higher osmolarities. Minimisation of acetic acid permeability could arise as a result of increased cytoplasmic membrane order brought about by, for example, sustained plasmolysis (Chapman *et al.*, in preparation: Results chapter 4), or enrichment with more highly ordered phospholipids such as cardiolipin (Chapman and Ross, in preparation: Results chapter 5).

One manner in which to test the rate of diffusion of acetic acid in response to increasing osmolarity is via the combined nuclear magnetic resonance (NMR) paramagnetic ion-induced line broadening / dynamic light scattering method of Xiang and Anderson (1995b). In this method, non-permeant paramagnetic ions (Pr^{3+}) can used to induce a shift in the NMR spectrum of C^{13}-labelled acetic acid external to cells, thus differentiating the extracellular acetic acid pool from the

intracellular acetic acid pool (Xiang and Anderson, 1995b). By this method, the relative permeability coefficients for acetic acid in response to osmolarity, corrected for cell volume by dynamic light scattering, could be determined and compared. By this method also, the relative effects of other environmental factors including pH and temperature on acetic acid permeability might also be able to be determined.

To determine the possibility of a rate-limiting region of the cell envelope (i.e. outer membrane, periplasm or cytoplasmic membrane) to acetic acid diffusion, consideration could be given to a comparison of permeability coefficients for whole cells, spheroplasts and protoplasts. Further, more controlled determinations of the effect of, for example, cardiolipin-enrichment on membrane permeability could be undertaken using liposome models. A commonly used model system for *E. coli* membranes is a mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) lipids, but recently Lopes *et al.* (2010) developed a ternary liposome model containing cardiolipin. Alternatively, studies could be undertaken to compare acetic acid permeability between wild-type and cardiolipin-deficient (e.g. *cls*$^-$) mutants.

8.3.2 Reduction of outer membrane damage by cell-solution interface changes

Bacteria possess a net negative surface charge at normal physiological pH and ionic strength, but tend to hold cations when placed into electrolyte solutions (James, 1991; *see* Sections 2.6.3 and 2.6.4). Low pH stress has been hypothesised to weaken the outer membrane by protonation of anionic groups of LPS (Alakomi *et al.*, 2000) (*see* Section 2.4.5). Despite observations in this thesis that protection against acetic acid inactivation occurs in the presence of both ionic and non-ionic osmolytes, it is tempting to speculate on a role for competitive interactions between H^+ and Na^+ cations at the cell-solution interface in protection of cells by NaCl against acetic acid.

For *E. coli*, the inflection points in the non-monotonic survival response were dependent on pH, with higher NaCl concentrations demonstrated to be more protective under lower pH conditions (compare panels a, b and c of Figure A2, Appendix A). At ~ 95 picometers, the ionic radius of Na^+

is less than half that of H^+, at ~ 208 picometers (Aylward and Findlay, 1974). Therefore, it is hypothesised that the competitive interaction of Na^+ with LPS may be less damaging to the outer membrane than is the interaction of H^+.

One method by which to test this hypothesis is by a quantitative comparison of LPS release from the outer membrane in the presence of acetic acid at different osmolarities. This method could also be used to test whether storage temperature changes the dynamics of loss of LPS from the outer membrane in the presence of acetic acid.

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10 Appendix A: Supplemental data

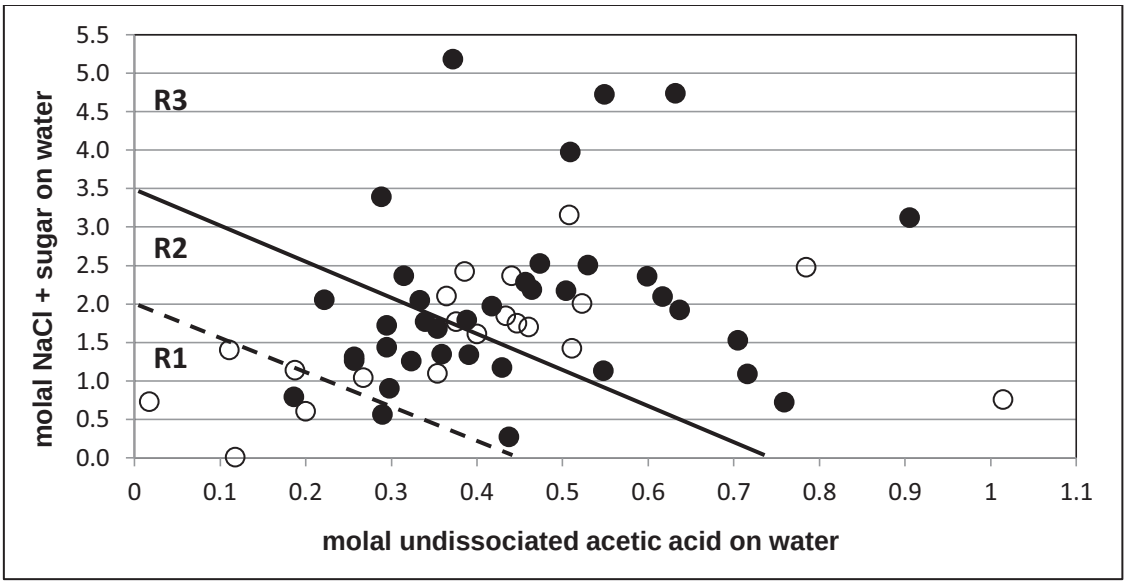

FIG A1. Comparison of surveyed, Australian-manufactured cold-filled sauce, dressing and mayonnaise formulations with formulations for which the CIMSCEE Code (CIMSCEE, 1992) may generate fail-dangerous predictions of microbiological safety (Chapman *et al.*, 2006). Details of surveyed formulations are provided in Chapman *et al.* (2010; Table 2). Some formulations falling into Region 2 (R2) with pH ≤ 3.5 (solid-fill; pH > 3.5 are unfilled), for which CIMSCEE (1992) predicted at least a 3-$\log_{10}$ reduction in 72h, were experimentally determined by Chapman *et al.* (2006) not to achieve this rate of inactivation (*see* Figure 1 in Chapman *et al.* (2006)).

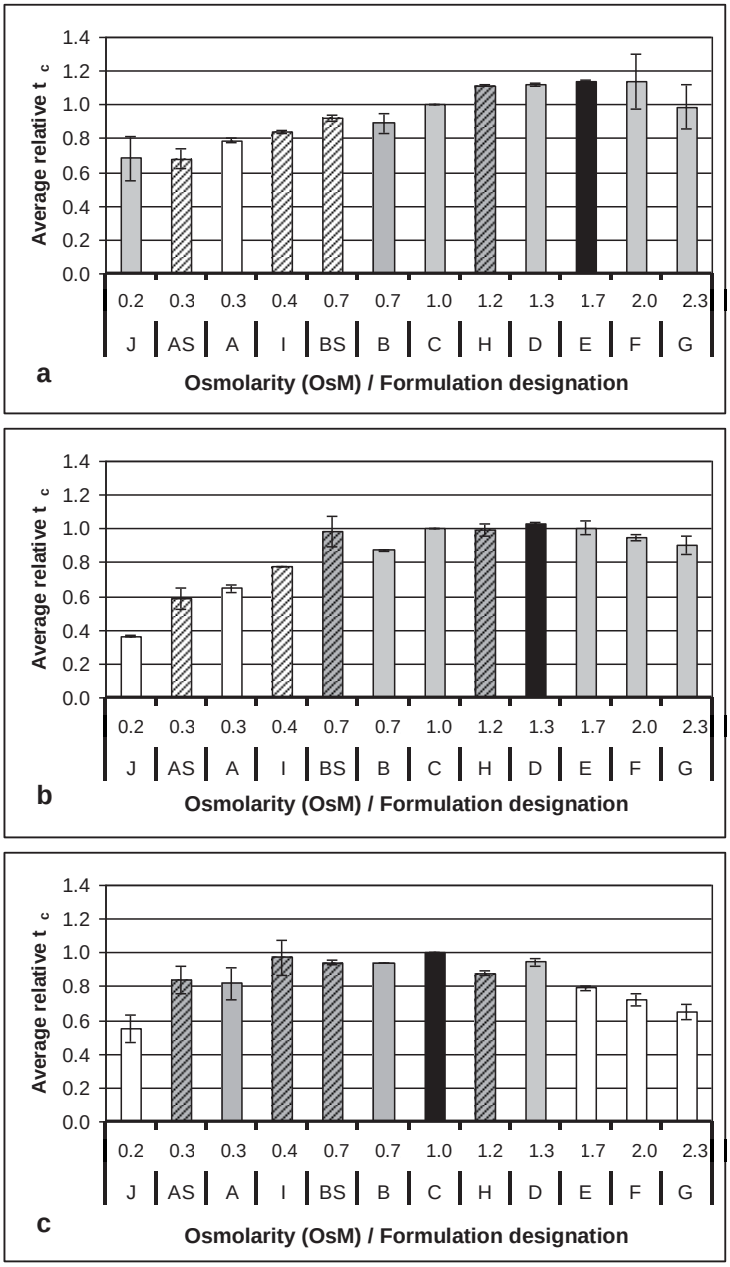

FIG. A2. Analysis of variance of average relative lag time, At_{cR} (normalised against At_c for 1.0 OsM, Formulation C), for *E. coli* (FRRB 2699) in response to osmolarity at pH (a) 3.6, (b) 3.8, (c) 4.0. Error bars indicated standard deviation (n=2). Maximum At_{cR} at each pH is indicated as solid black bar; At_{cR} values represented as shaded bars are not significantly different ($\alpha = 0.05$) from maximum At_{cR} (for (C), significance is compared against formulations B and D, 0.7 and 1.3 OsM, respectively, as maximum At_{cR} values is found at 1.0 OsM (Formulation C), used for normalising other results). Hatching indicates formulations containing sucrose. For details of methodology refer to Chapman and Ross (2009).

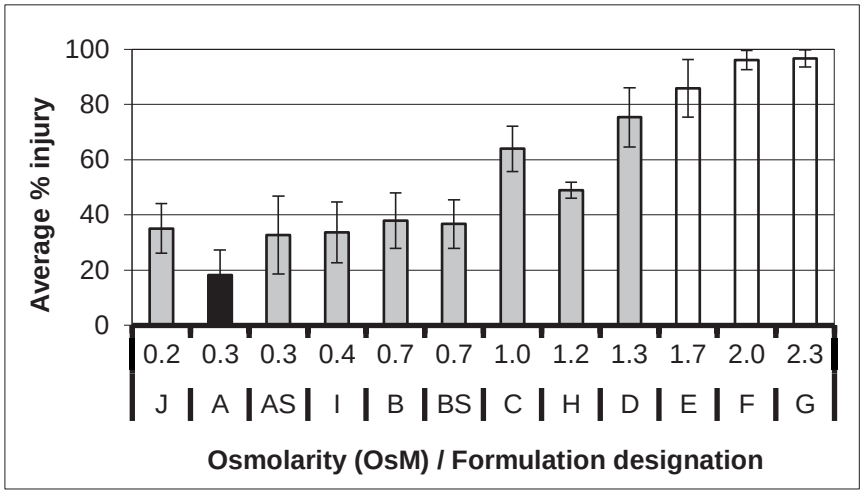

FIG. A3. Average % injury of *S. enterica* (FRRB 2742) at pH 4.0 at different osmolarities after 2 h at 23°C, as assessed by sensitivity to crystal violet assay. Error bars indicate standard deviation (n = 2). Black fill indicates formulation with minimum % injury, from among formulations containing NaCl only (i.e. excluding formulations AS, I, BS and H containing sucrose); grey fill indicates average % injury is not significantly different ($p > 0.05$) to minimum % injury; no fill indicates average % injury is significantly different ($p < 0.05$) to minimum % injury. For details of methodology refer to Chapman *et al.* (in preparation).

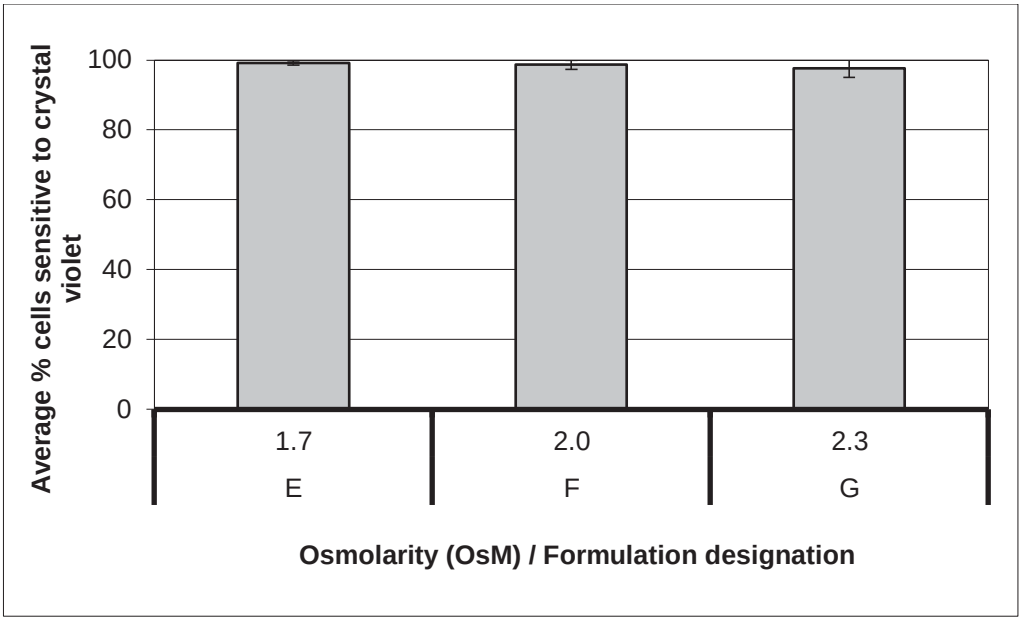

FIG. A4. Average % *S. enterica* (FRRB 2742) cells sensitised to crystal violet at neutral pH and high osmotic pressures after 2 h at 23°C. Error bars indicate standard deviation (n = 2). For details of methodology refer to Chapman *et al.* (in preparation).

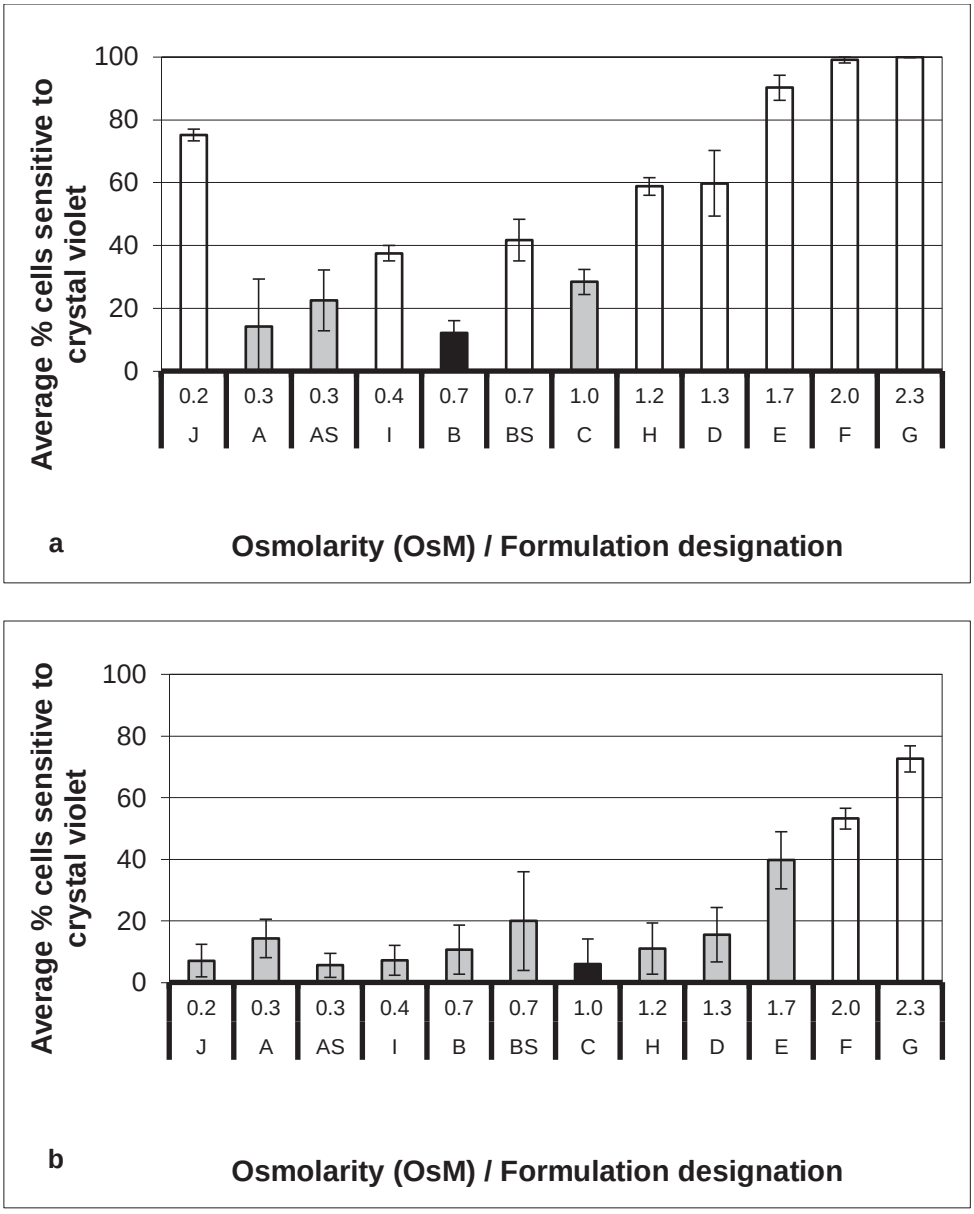

FIG. A5. Average % injury of *E. coli* (FRRB 2699) at pH 3.6 (a) and pH 4.0 (b) at different osmolarities after 2 h at 23°C, as assessed by sensitivity to crystal violet. Error bars indicate standard deviation (n = 2). Black fill indicates formulation with minimum % injury, from among formulations containing NaCl only (i.e. excluding formulations AS, I, BS and H containing sucrose); grey fill indicates average % injury is not significantly different ($p > 0.05$) to minimum % injury; no fill indicates average % injury is significantly different ($p < 0.05$) to minimum % injury. For details of methodology refer to Chapman *et al.* (in preparation).

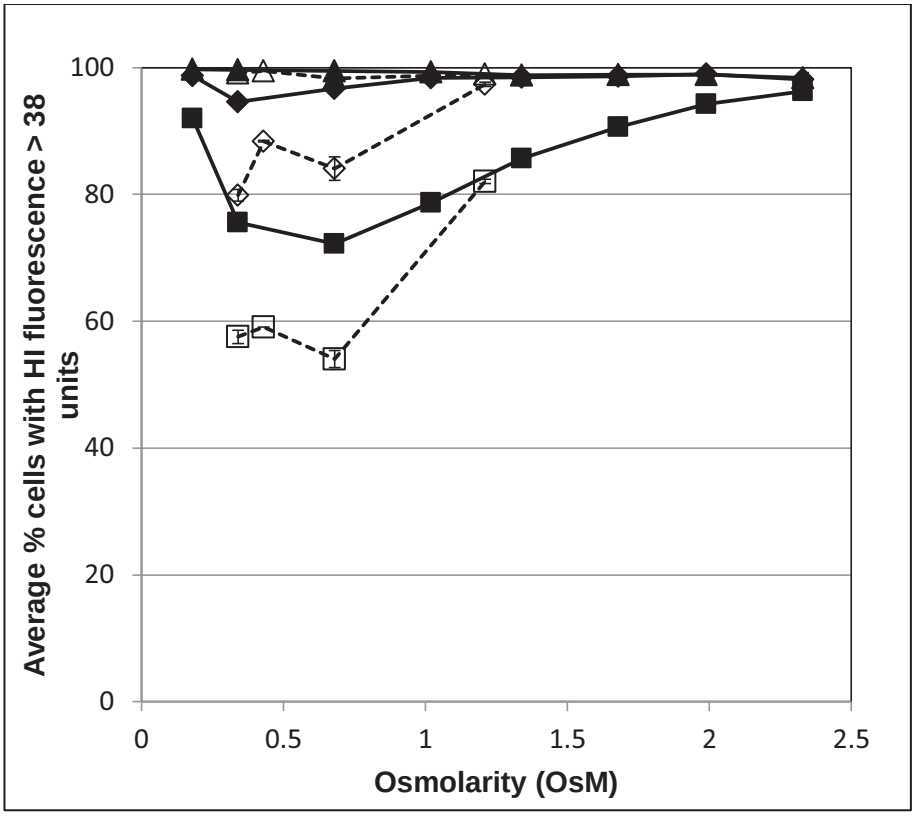

FIG. A6. Average % of *S. enterica* (FRRB 2742) cells with hexidium iodide fluorescence > 38 units after exposure to acetic acid at pH 4.0 for 2 (squares), 24 (diamonds), or 72 (triangles) at 23°C versus calculated osmotic pressure (at 23°C). Filled shapes and solid lines indicate formulations containing NaCl only; unfilled shapes and dotted lines indicate formulations containing sucrose and NaCl. Error bars indicate standard deviation (n = 2). For details of methodology refer to Chapman *et al.* (in preparation).

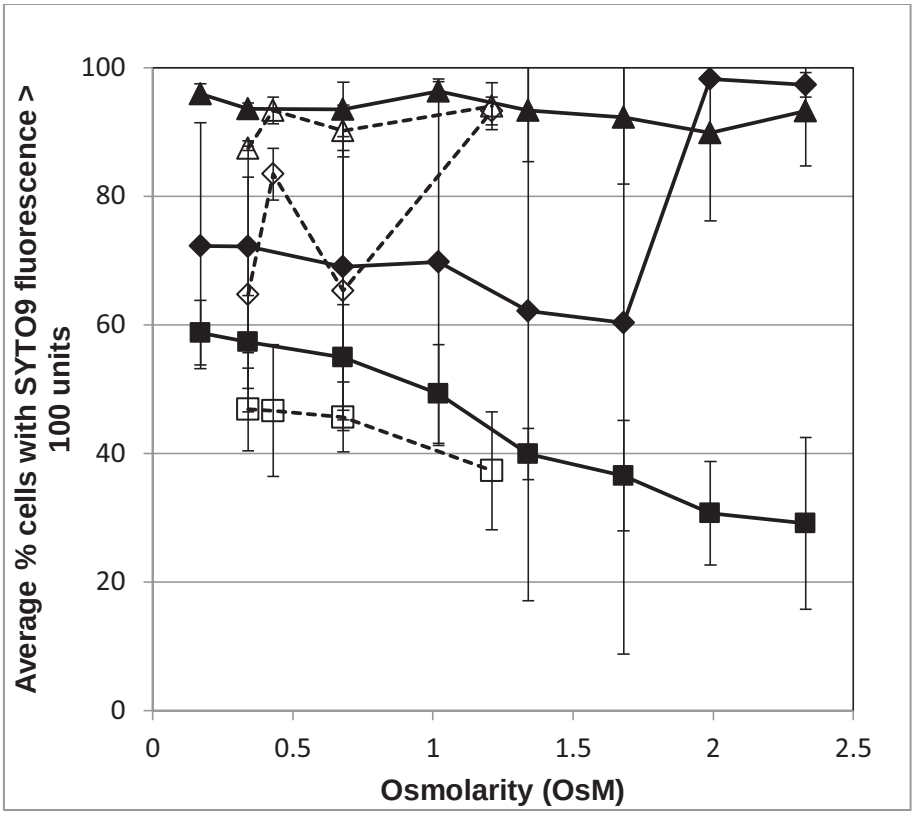

FIG. A7. Average % of *S. enterica* (FRRB 2742) cells with SYTO® 9 fluorescence > 100 units after exposure to acetic acid at pH 4.0 for 2 (squares), 24 (diamonds), or 72 (triangles) at 23°C versus calculated osmotic pressure (at 23°C). Filled shapes and solid lines indicate formulations containing NaCl only; unfilled shapes and dotted lines indicate formulations containing sucrose and NaCl. Error bars indicate standard deviation (n = 2). For details of methodology refer to Chapman *et al.* (in preparation).

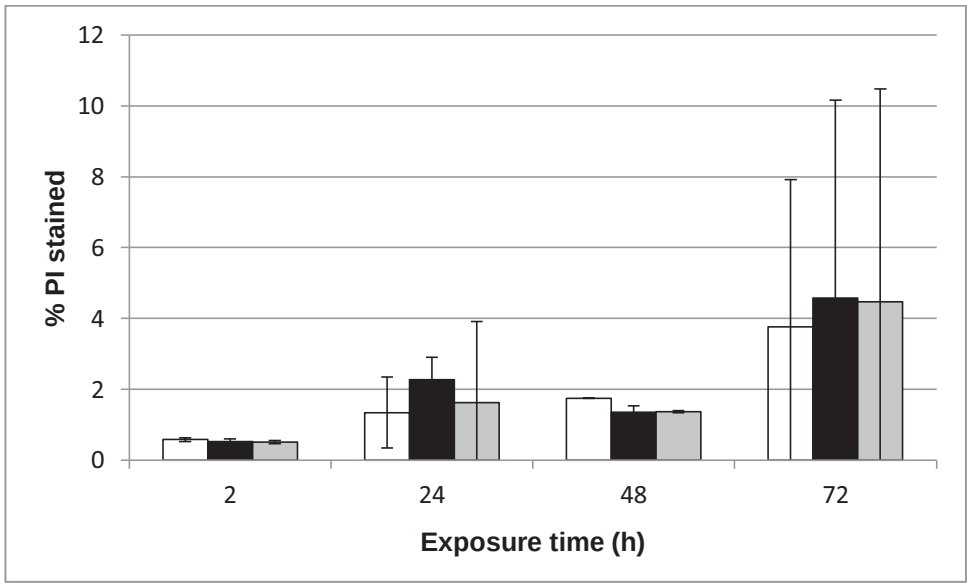

FIG. A8. Effect of osmolarity in the presence of acetic acid on temporal damage to *S. enterica* (FRRB 2742) at pH 4.0 and 23°C, assessed by propidium iodide (PI) (dual stain procedure). Unfilled = 1.68 OsM (Formulation E), black = 1.99 OsM (Formulation F), grey = 2.33 OsM (Formulation G). Error bars indicate standard deviation (n = 2). For details of methodology refer to Chapman *et al.* (in preparation).

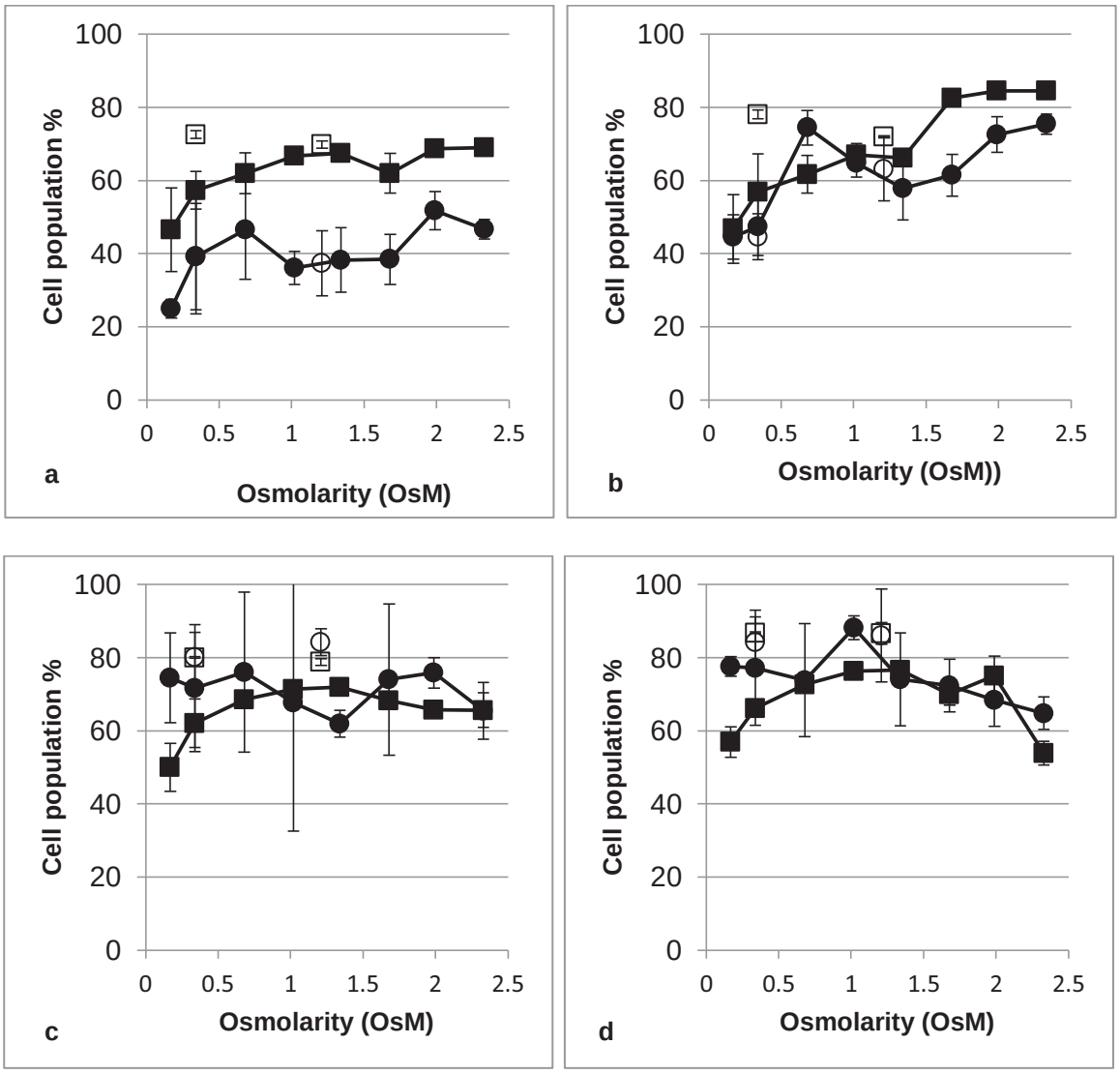

FIG. A9. Average % of *E. coli* cells with reduced membrane potential measured using nonyl acridine orange (circles) or $DiOC_2(3)$ (squares) after (a) 6, (b) 24, (c) 48 and (d) 72 h exposure to acetic acid at different osmolarities adjusted using NaCl alone (filled symbols), or NaCl + sucrose (unfilled symbols). Error bars indicate standard deviation (n = 3 for NAO, n = 2 for $DiOC_2(3)$). For details of methodology refer to Chapman and Ross (in preparation).

11 Appendix B: declarations of co-authorship

11.1 Manuscript 1

Declaration of co-authorship	
Article title:	Salt, alone or in combination with sucrose, can improve the survival of *Escherichia coli* O157 (SERL 2) in model acidic sauces
Co-authors:	Nancy Jensen, Tom Ross, Martin Cole

Evaluation scale:	
1	has contributed to this work (0-33%)
2	has made substantial contribution to this work (34-66%)
3	has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation / identification of the specific problem that needs to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	3
2. Planning of the experiments and methodology design, including selection of methods and method development.	3
3. Involvement in the experimental work	3
4. Presentation, interpretation and discussion in a journal format of the obtained data	3
Overall contribution	3

Signatures of the co-authors	
Nancy Jensen	
Tom Ross	
Martin Cole	

11.2 Manuscript 2

Declaration of co-authorship	
Article title:	*Escherichia coli* and *Salmonella enterica* are protected against acetic acid, but not hydrochloric acid, by hypertonicity
Co-authors:	Tom Ross

Evaluation scale:	
1	has contributed to this work (0-33%)
2	has made substantial contribution to this work (34-66%)
3	has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation / identification of the specific problem that needs to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	**3**
2. Planning of the experiments and methodology design, including selection of methods and method development.	**3**
3. Involvement in the experimental work	**2**
4. Presentation, interpretation and discussion in a journal format of the obtained data	**3**
Overall contribution	**3**

Signatures of the co-authors
Tom Ross

11.3 Manuscript 3

Declaration of co-authorship	
Article title:	Contemporary formulation and distribution practices for cold-filled products: Australian industry survey and modelling of published pathogen inactivation data
Co-authors:	Katherine Scurrah, Tom Ross

Evaluation scale:	
1	has contributed to this work (0-33%)
2	has made substantial contribution to this work (34-66%)
3	has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation / identification of the specific problem that needs to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	**3**
2. Planning of the experiments and methodology design, including selection of methods and method development.	**2**
3. Involvement in the experimental work	**2**
4. Presentation, interpretation and discussion in a journal format of the obtained data	**2**
Overall contribution	**2**

Signatures of the co-authors		
Katherine Scurrah		
Tom Ross		

11.4 Manuscript 4

Declaration of co-authorship	
Article title:	*Escherichia coli* outer and cytoplasmic membrane changes during exposure to acetic acid, in response to exposure time, osmolytes, temperature and pH
Co-authors:	Lynne Turnbull, Cynthia Whitchurch, Tom Ross

Evaluation scale:	
1	has contributed to this work (0-33%)
2	has made substantial contribution to this work (34-66%)
3	has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation / identification of the specific problem that needs to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	**3**
2. Planning of the experiments and methodology design, including selection of methods and method development.	**3**
3. Involvement in the experimental work	**3**
4. Presentation, interpretation and discussion in a journal format of the obtained data	**3**
Overall contribution	**3**

Signatures of the co-authors
Lynne Turnbull
Cynthia Whitchurch
Tom Ross

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Declaration of co-authorship	
Article title:	Cardiolipin production by *Escherichia coli* and *Salmonella enterica* in response to time, osmolytes, temperature and pH during exposure to acetic acid, and changes in membrane potential and fluidity
Co-authors:	Tom Ross

Evaluation scale:	
1	has contributed to this work (0-33%)
2	has made substantial contribution to this work (34-66%)
3	has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation / identification of the specific problem that needs to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	**3**
2. Planning of the experiments and methodology design, including selection of methods and method development.	**3**
3. Involvement in the experimental work	**3**
4. Presentation, interpretation and discussion in a journal format of the obtained data	**3**
Overall contribution	**3**

Signatures of the co-authors	
Tom Ross	

12 Appendix C: other research outputs

Conference presentations arising from this thesis:

Chapman, B., Turnbull, L., Whitchurch, C., Ross, T. Single cell resolution of changes to *Escherichia* coli membrane structure and function in the presence of acetic acid at a range of NaCl concentrations. 112th General Meeting of the American Society for Microbiology, San Francisco, June 2012

Chapman, B., Scurrah, K., Ross, T. Product formulation and food safety hurdles. Australian Institute of Food Science and Technology (AIFST) 13th Food Microbiology Conference, Melbourne, March 2009

Scurrah, K., Ross, T., Chapman, B. Microbial safety of mayonnaises, sauces and dressing manufactured in Australia, Australian Institute of Food Science and Technology (AIFST) 40th Annual Convention, Melbourne, June 2007

Chapman, B. Interfacing science at the microbial membrane. Australian Institute of Food Science and Technology (AIFST) 12th Food Microbiology Conference, Sydney, February 2006

Other communications arising from this thesis:

Scurrah, K., Chapman, B., Ross, T. Traditional food safety concepts: challenges and solutions. A case study with cold-filled acid sauces. Presentation at Food Safety and Food Manufacturing Factory Focus Workshop hosted by the Victorian Food Industry and High Performance Consortium and the Australian Food Safety Centre of Excellence, Shepparton, Victoria, 17th May 2007

Chapman, B. 2006. Safety of cold filled sauces. Food Aust. **58:**582-583.